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Decoding E-cadherin glycans functions in cancer: from functional glycomics to clinical applications.

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The author of this thesis declares that, in accordance with "nº 2, alinea a, do Art.º 31º do Decreto-Lei nº 230/2009", afforded a major contribution to the design and technical execution of the work, interpretation of the results and manuscript preparation resulting in the following accepted and in preparation articles:

Scientific publications

- Julio Cesar Madureira de-Freitas-Junior*, **Sandra Carvalho***, Ana M. Dias, Patricia Oliveira, Joana Cabral, Raquel Seruca, Carla Oliveira, José Andrés Morgado-Diaz, Celso A. Reis, Salomé S. Pinho (2013). Insulin/IGF-I Signalling Pathways Enhances Tumour Cell Invasion through Bisecting GlcNAc N-glycans Modulation. An Interplay with E-cadherin. PLoS One. Nov 25; 8 (11):e811579. * *These authors contributed equally to this work.*

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- **Sandra Carvalho***, Tiago Oliveira*, Markus Bartels, Eiji Miyochi, Michael Pierce, Naoyuki Taniguchi, Fátima Carneiro, Raquel Seruca, Sabine Strahl, Celso A. Reis, Salomé S. Pinho (2015) O-mannosylation and N-glycosylation: two coodinated mechanisms regulating critical functions of E-cadherin in cancer. (In preparation). * *These authors contributed equally to this work.*

- Pinho SS, **Carvalho S**, Marcos-Pinto R, Magalhães A, Oliveira C, Gu J, Dinis-Ribeiro M, Carneiro F, Seruca R, Reis CA. (2013) Gastric cancer: adding glycosylation to the equation. Trends Mol Med. 2013 Nov; 19(11):664-76. doi: 10.1016/j.molmed.2013.07.003. Epub 2013 Aug 8.

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Resumo

Cancro gástrico é uma doença agressiva com um elevado impacto na saúde mundial, devido ao diagnóstico tardio dos pacientes. Os eventos moleculares subjacentes ao processo de carcinogénese gástrica têm vindo a ser abordados de modo a identificar biomarcadores moleculares específicos para a deteção precoce do carcinoma gástrico. O cancro gástrico do tipo difuso, caracterizado por uma reduzida adesão intercelular, tem vindo a ser descrito como estando estritamente relacionado com a desregulação da proteína supressora de invasão, E-caderina. O comprometimento das funções biológicas da E-caderina resulta na perda da adesão celular e no aumento do potencial de invasão e metastização. E-caderina é uma glicoproteína que é modificada por glicosilação pós-translacionalmente e cujas modificações têm forte efeito na doença neoplásica. De facto, a transformação maligna está fortemente associada com padrões alterados de glicosilação na superfície das células tumorais.

A N-glicosilação da E-caderina mediada por GnT-III confere-lhe uma estabilidade na membrana celular o que conduz às suas funções adesivas adequadas. Esta glicoforma da E-caderina também regula a atividade da GnT-III por um mecanismo bidirecional o que contribui para a supressão de invasão e metastização. Neste estudo, demonstramos que este mecanismo é comprometido com a ativação da via de sinalização insulina / IGF-I no processo de invasão de células tumorais. Por outro lado, E-caderina pode também ser sujeita a N- glicosilação mediada por GnT-V o que compromete a adesão célula-célula. Pacientes com cancro gástrico exibem uma expressão aberrante da E-caderina especificamente modificada com estruturas β1,6 GlcNAc. Demonstramos que estes N-glicanos deletérios estão presentes especificamente no local de N-glicosilação Asn-554 da E-caderina, sendo o local chave para a sua desregulação funcional nas células tumorais gástricas. Além disso, também verificamos que o perfil de O-manosilação da E-caderina, descrito recentemente como sendo crucial para a adesão célula-célula, encontra-se comprometido num contexto de cancro gástrico com implicações nas suas funções biológicas. O impedimento da ocupação do local Asn-554 da E-caderina com N-glicanos β1,6 GlcNAc potencializa o perfil de O-manosilação da E-caderina, estando assim associado com a recuperação das suas funções biológicas. Adicionalmente, verifica-se a existência de um mecanismo coordenado entre o perfil de O-manosilação proteico e a N-glicosilação mediada por GnT-V em pacientes com cancro gástrico do tipo difuso. Assim sendo, a descrição do perfil de glicosilação da E-caderina em cancro gástrico pode contribuir para potenciais glico- biomarcadores de diagnóstico precoce do cancro gástrico do tipo difuso, e para o desenvolvimento de potenciais alvos terapêuticos.

Abstract

Gastric cancer is an aggressive disease with a high impact on global health due to the late diagnosis of gastric cancer patients. The molecular events underlying gastric cancer process have been addressed in order to identify molecular biomarkers specifics for the early detection of gastric carcinoma. Diffuse gastric cancer, characterized by a reduced intercellular adhesiveness, has been strongly correlated with the dysregulation of the invasion suppressor protein, E-cadherin. The impairment of E-cadherin biological functions results in loss of cell adhesion and increased cell invasion and metastatic potential. E-cadherin is a glycoprotein that is post-translationally modified by glycosylation which may exert a powerful effect on the outcome of neoplastic disease. In fact, malignant transformation is strongly associated with altered glycosylation patterns on the surface of cancer cells.

E-cadherin N-glycosylation mediated by GnT-III confers a stability to the E-cadherin at the cell membrane leading to the proper adhesive functions. Moreover, this E-cadherin glycoform also regulates the GnT-III activity by a bidirectional crosstalk which contributes to the suppression of tumour invasion and metastasis. In this study, we have shown that this interplay is compromised with the activation of the insulin/IGF-I signalling pathway in the process of tumour cell invasion. E-cadherin may also undergo GnT-V-mediated N-glycosylation that compromises the E-cadherin-mediated cell-cell adhesion. Human gastric cancer patients display an aberrant expression of E-cadherin specifically modified with the β1,6 GlcNAc branched N-glycans. Here, we have demonstrated that these deleterious N-glycans are present specifically in the E-cadherin N-glycosylation site Asn-554, being the key site for the functional dysregulation of E-cadherin in gastric tumour cells. Furthermore, we have shown that the O-mannosylation profile of E-cadherin, recently described crucial in cell-cell adhesion, is impaired in a gastric cancer context with implications in its biological functions. Precluding the occupancy of E-cadherin Asn-554 with β1,6 GlcNAc branched N-glycans potentiates the O-mannosylation profile of E-cadherin being associated with the recovery of the proper E-cadherin biological functions. In addition, we also have demonstrated the existence of a coordinated interplay between protein O-mannosylation and GnT-V-mediated N-glycosylation in human diffuse gastric cancer patients. Hence, the disclosure of the E-cadherin glycosignature in gastric cancer may contribute to potential glycobiomarkers for the early diagnosis of diffuse gastric cancer, and to the development of potential therapeutic targets.

Abbreviations

a.a – amino acids
ALG - asparagine linked glycosylation
Asn - asparagine
CA19-9 – cancer antigen 19-9
CBD - Catenin binding domain
CKII - Casein kinase II
CMD - congenital muscular dystrophies
CNX - calnexin
CRT - calreticulin
Dol-P - dolichol phosphate
Dpm1 - GDP-Man: Dol-P mannosyltransferase
EBV - Epstein-Barr virus
E-cadherin- epitelial cadherin
ECM - extracellular matrix
EMT- Epithelial-mesenchymal transition
ER - Endoplasmic reticulum
ERAD - ER- associated degradation
Fuc – fucose
FUT8 - α1,6-fucosyltransferase
Gal - galactose
Gal - galactose
GalNAc - N-acetylgalactosamine
Glc - glucose
GlcNAc - N-acetylglucosamine
GlcNAcT-I (or GnT-I) - N-acetylglucosaminyltransferase I
GlcNAcT-II (or GnT-II) - N-acetylglucosaminyltransferase II
GlcNAcT-III (or GnT-III) - N-acetylglucosaminyltransferase III
GlcNAcT-IV (or GnT-IV) - N-acetylglucosaminyltransferase IV
GlcNAcT-V (or GnT-V) - N-acetylglucosaminyltransferase V
GlcNAcT-VI (or GnT-VI) - N-acetylglucosaminyltransferase VI
GnT-IX/ GnT-Vb - β1,6-N-acetylglucosaminyltransferase-IX/Vb
GSK-3β - glycogen synthase kinase-3β
H. pylori - Helicobacter pylori
HAV - Histidine-Alanine-Valine

HCC - hepatocellular carcinoma
HDGC - Hereditary diffuse gastric carcinoma syndrome
IARC - International Agency for Research on Cancer
IGF-IR - IGF-I receptor
JMD - Juxtamembrane domain
LOH - Loss of heterozygosity
Man - mannose
MEB - Muscle-eye-brain
MGAT5 - mannoside acetylglucosaminyltransferase 5 gene
miRNAs - MicroRNAs
Neu5Ac- sialic acid
OST - oligosaccharyltransferase
PIPKIγ - type I gamma phosphatidylinositol phosphate kinase
PMTs - protein O-mannosyltransferases
polyLacNAc - poly-N-acetyllactosamine
POMGnT1 - β-1,2-N-acetylglucosaminyltransferase 1
POMGnT2 - β-1,4-N-acetylglucosaminyltransferase 2
POMK - protein O-mannose kinase
POMT1 - protein O-mannosyltransferase 1
POMT2 - protein O-mannosyltransferase 2
PP2A - protein phosphatase 2A
RPTPβ - receptor tyrosine phosphatase β
RTK - receptors tyrosine kinase
Ser - serine
TGN - trans-Golgi network
Thr - threonine
TJ - tight junctions
UGGT - UDP-Glc: glycoprotein glucosyltransferase
UPR - unfolded protein response
WHO - World Health Organization
WWD - Walker-Warburg syndrome
α-DG - α- dystroglycan

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Chapter I

General Introduction

Gastric Cancer

Epidemiology and Aetiology

Gastric cancer is an aggressive disease with a daunting impact on global health. Gastric cancer remains the sixth most common type of cancer, and is the fourth leading cause of cancer-related death worldwide, after lung, breast and liver cancer [1]. Despite the decline in incidence over the past few decades, gastric cancer continues to present a major clinical challenge due to late diagnosis of most gastric cancers since survival is highly dependent on the stage at which the tumour is diagnosed [2].

Large differences in gastric cancer incidence exists between continents, being highest incident rates found in Asia, followed by Europe, Central and South America, North America and Africa [3, 4]. Even within a given geographical area, certain ethnic groups have significantly higher risk of disease [5]. The risk of developing gastric cancer increases with age [6]. The male-to- female ratio in incidence and mortality is about 2:1 [7].

Gastric cancer is a multifactorial disease: infectious, environmental, and host-related factors may interact favouring the development of tumour [8]. Chronic infection with *Helicobacter pylori* (*H. pylori*), classified as a class I carcinogen according the International Agency for Research on Cancer (IARC), has been implicated in gastric carcinogenesis [9-11]. It is estimated that 50% of the world´s population is infected with *H. pylori* [12]. Another infectious agent associated with gastric cancer is the Epstein-Barr virus (EBV) [13]. Regarding environmental and lifestyle factors, tobacco smoking [14], low socioeconomic status, alcohol and meat consumption [15], diet rich in salt and poor in fruits and vegetables [16, 17], and high body mass index [18] are closely linked to increased risk of gastric cancer. At last, molecules involved in adhesion of the bacteria [19] as well as host genetic polymorphisms from diverse molecular pathways [20] are also associated with progression of the gastric carcinogenesis pathway. Genome-wide association studies have been largely used to evaluate single nucleotide polymorphisms simultaneously opening new avenues in cancer research [21].

Histological classification

Gastric cancer is a complex and heterogeneous disease with different morphologies, histogenesis and molecular backgrounds [22, 23]. The major histological type of gastric cancer is adenocarcinoma, which originates from the glandular epithelium and accounts for 90% to 95% of all gastric malignancies [24]. Several gastric cancer classifications systems have been proposed over the past decades: World Health Organization (WHO) [25] and the Lauren´s classification [26]. The 2010 WHO system recognizes five major histologic patterns of gastric cancer: tubular, papillary, mucinous, poorly cohesive (including signet ring cell carcinoma), and mixed carcinomas [25, 27]. The Lauren´s criteria, which is the most commonly used classification, describes two main histological subtypes, the intestinal subtype and diffuse subtype gastric adenocarcinoma, which display distinct epidemiologic, morphological and molecular features [26, 28] (Figure 1).

The intestinal subtype of gastric cancer represents nearly 70% of the cases, being more frequently diagnosed in older male patients [29]. It is more likely to be sporadic than inherited [30]. Histologically, it is characterized by glandular architecture with cells displaying cohesiveness and various degrees of differentiation [31]. The main carcinogenic event associated with intestinal subtype cancer is *H. pylori* infection which leads to a sequence of histological lesions (known as Correa´s cascade) that culminate in a malignant lesion [32]. The cascade of events corresponds to a gradual and multistep progression from chronic gastritis to chronic atrophy to intestinal metaplasia to dysplasia and carcinoma [33]. At the molecular level, intestinal gastric cancer is associated with overexpression of c-met oncogene [34], K-ras mutations [35], loss-of-function of tumour suppressive genes TP53 [36] and APC [37], among others.

In contrast to the intestinal subtype, diffuse gastric cancer is generally diagnosed in younger patients [5]. The diffuse subtype of gastric cancer develops without precancerous lesions; it is characterized by poorly cohesive cells with little or no gland formation displaying a worse prognosis than the intestinal type [38, 39]. Diffuse gastric tumours have an important mechanism of carcinogenesis that is through defective intercellular adhesions, mainly resulting from E-cadherin dysregulation [38]. Abnormal E-cadherin expression can occur through biallelic inactivation of its related gene *CDH1* via germline or somatic mutations [40], loss of heterozygosity (LOH) [41, 42], epigenetic silencing of gene transcription through *CDH1* promoter hypermethylation [43, 44], transcriptional silencing that target *CDH1* promoter [45] or by alteration of E-cadherin glycosylation [46, 47]. *CDH1* inactivation occurs in early stages of diffuse type tumour development, whereas in intestinal type tumours it seem to take place in relatively late stages of carcinogenesis. Importantly,

CDH1 germline mutations characterizes the hereditary diffuse gastric carcinoma syndrome (HDGC) [48-50].

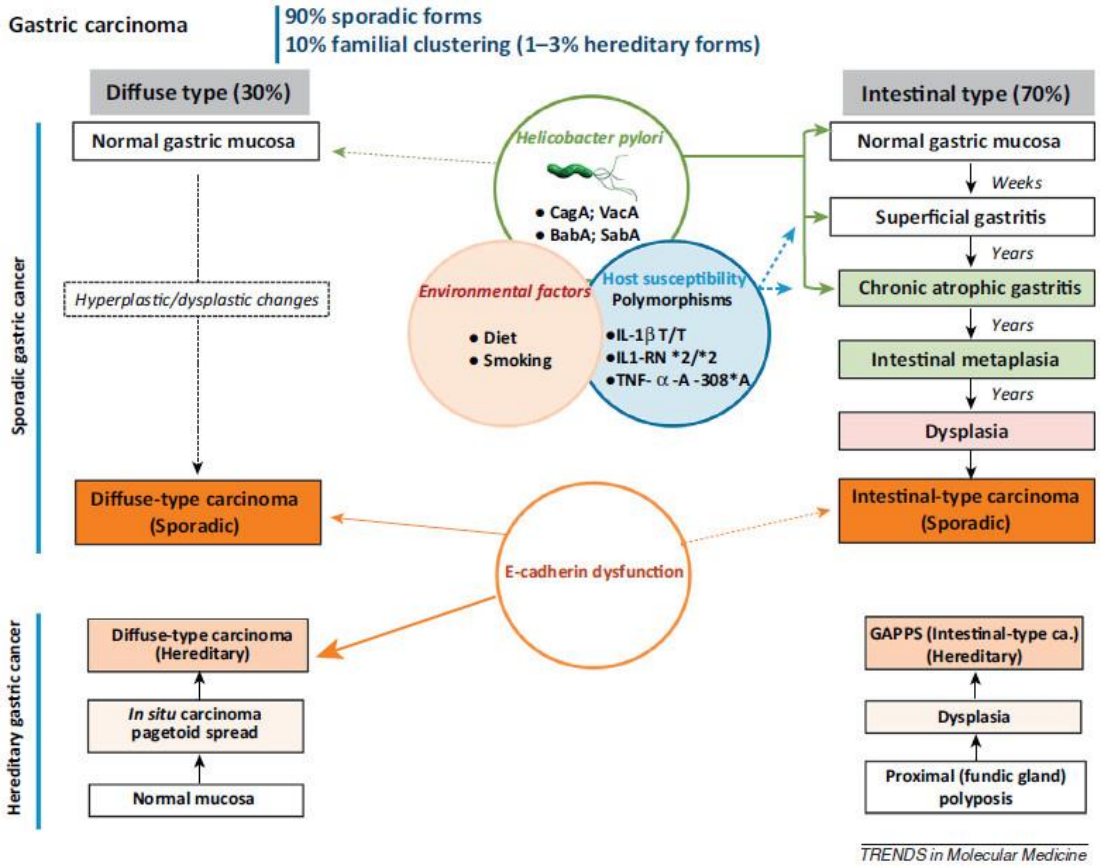

Figure 1 - Clinicopathological profiles and epidemiological settings of the two main histological subtypes of gastric cancer: intestinal subtype and diffuse subtype. Adapted from [47].

E-cadherin

Cadherins constitute a large superfamily of transmembrane glycoproteins that mediate specific cell-cell adhesion in a calcium-dependent manner [51]. The family is widespread in normal tissues but the individual members display pronounced tissue specificity [52]. The cadherin superfamily is mainly composed by “classical” cadherins of type 1, closely related cadherins of type II, desmossomal cadherins, protocadherins, and a variety of cadherin-related molecules [53, 54].

Classical cadherins were the first subtype of the cadherin superfamily identified in vertebrates [55-58]. Cadherins form primarily homophilic cell-cell interactions at the adherens junctions, and appear to modulate adhesion through dynamic interactions with the actin cytoskeleton [54, 59]. Epithelial cadherin (E-cadherin) is considered the

prototypical member of the cadherin superfamily, being identified in 1977 by Takeichi as a surface protein with Ca^{2+}-dependent cell-cell adhesion properties [60]. E-cadherin is expressed primarily in epithelial cells, where it localizes at the basolateral surface of the epithelial junctional complex- the adherens junctions [59].

The human *CDH1* gene, encoding E-cadherin, is situated in the long arm of chromosome 16, within the locus 16q22.1 [61, 62]. *CDH1* gene is organized into 15 introns and 16 exons, which are translated into protein comprising 882 aminoacids (a.a). The signal peptide corresponds to the first 27 a.a (exons 1-2), and is followed by the precursor peptide (exon 2-4) (Figure 2A) [42, 63, 64].

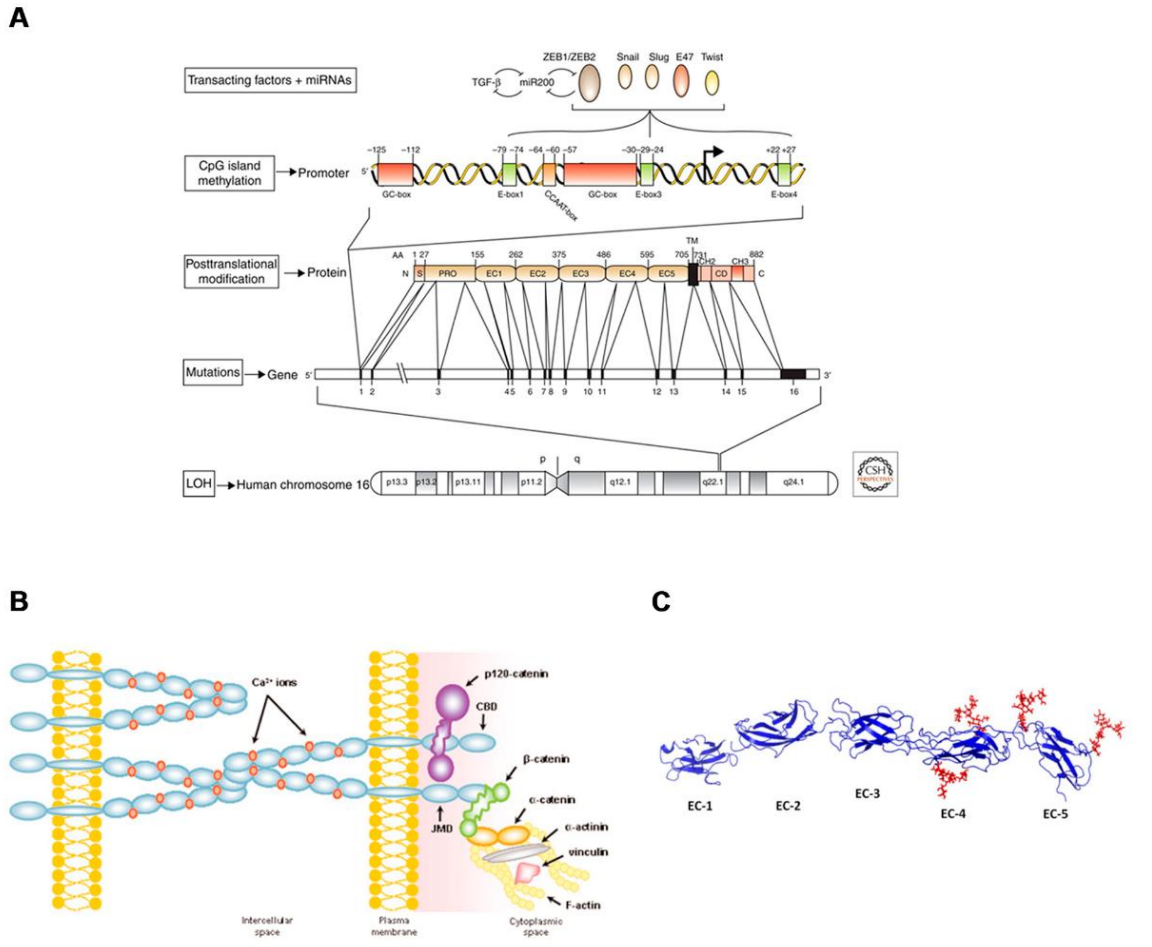

Figure 2 - Schematic representation of (A) different levels at which E-cadherin expression is regulated in human tumours, [42] (B) classical cadherin-catenin complex [65], and (C) three-dimensional structure of the extracellular domain (EC1–EC5) of E-cadherin combined with the representation of the four potential N-glycosylation sites [46].

The immature form of E-cadherin (the precursor protein) is a polypeptide composed by a propeptide sequence of about 130 a.a and a mature polypeptide of about 728 a.a (Figure 2A) [42]. The propeptide corresponds to a short signal sequence for import into the endoplasmic reticulum (ER) where undergoes cytoplasmic trimming. Following this trimming process, the mature E-cadherin is routed towards the basolateral surface of epithelial cells [64]. E-cadherin mature protein is organized in three major structural domains: an N-terminal ectodomain of about 550 a.a comprising five tandemly repeated subdomains (EC1-EC5), a single transmembrane domain, and a short cytoplasmic domain (C-terminal) of about 150 a.a [46, 64].

Extracellular domain of E-cadherin

The cell-cell adhesion mediated by E-cadherin is achieved through homophilic interactions of the extracellular domain of E-cadherin molecules. The N-terminal domain EC1 has been identified to correspond to the adhesive binding site [66]. EC1 contains a Histidine-Alanine-Valine (HAV) sequence which is thought to be essential for the process of cell-cell adhesion [67]. According to the literature, cadherins may form both lateral (*cis*) and adhesive (*trans*) dimers on the cell surface though distinct interactions involving EC1 or EC1-EC2 domains [68-70]. The cadherin molecules on the cell surface establish lateral or *cis*- interaction and then among adjacent cells form *trans* adhesive bonds, forming zipper-like structures [71]. *Trans*-interactions on opposing cell surfaces result in weak cell-cell adhesion, but strong cell-cell adhesion develops during lateral clustering of E-cadherin [72]. The five EC domains are rigidified by coordinating Ca^{2+} ions between any two consecutive EC domains [66, 73] and this binding of Ca^{2+} is essential to confer resistance of the extracellular region to proteolytic degradation [56, 74].

Intracellular domain of E-cadherin

Further strengthening of cell-cell adhesion requires subsequent linkage to the cytoskeleton that is accomplished through the interaction of the E-cadherin cytoplasmic domain with cytoplasmic proteins called catenins (Figure 2B) [66]. The cytoplasmic domain is subdivided into the juxtamembrane domain (JMD) that provides a specific binding site for p120-catenin and p120-related proteins [75, 76], and the catenin binding domain (CBD) which specifically binds to β-catenin and plakoglobin [77].

Interaction of β-catenin to E-cadherin is required for transportation of the newly synthesized E-cadherin protein from the ER to the cell membrane [78]. β-catenin binds the

C–terminal cytoplasmic domain of E-cadherin in a phospho-regulated manner [79]. Phosphorylation of the three serine residues located in the cadherin cytoplasmic domain (S684, S686, S692) by casein kinase II (CKII) and glycogen synthase kinase-3β (GSK-3β) leads to increase in affinity of E-cadherin and β-catenin interaction. In contrast, tyrosine phosphorylation of β-catenin at Y489 and Y654 weakens interaction with cadherin [80]. In addition, β-catenin plays an important role in cell signalling pathways, such as in Wnt signalling [81, 82]. β-catenin functions as a transcriptional co-regulator, moving from the cytoplasm into the nucleus and cooperating with TCF/LEF transcription in order to activate the expression of numerous genes involved in migration and proliferation [83, 84]. Furthermore, β-catenin plays a key role in cadherin-adhesive function by acting as an adaptor for a range of cytoplasmic proteins which in turn interact with the actin cytoskeleton [72]. The best known is α-catenin which exists in either a monomeric or homo-dimeric state: monomeric α-catenin binds to the N-terminal portion of β-catenin while homo-dimeric α-catenin binds to actin filaments [72, 85]. Phosphorylation of β-catenin at Y142 disrupts binding to α-catenin [80].

E-cadherin stabilization at the cell membrane also occurs through the association with p-120-catenin that interacts with the highly conserved juxtamembrane domain of E-cadherin [75]. E-cadherin-p120-catenin interaction prevents the entrance of E-cadherin into degrading endocytic trafficking pathways or accelerates the recycling of internalized cadherin back to the plasma membrane [86]. The phosphorylation of p120-catenin increases binding affinity to E-cadherin [72, 87]. However, the loss of E-cadherin-p120 catenin interaction destabilizes cadherin localization at the cell membrane, inhibiting its accumulation at cell borders [88]. Moreover, the weakness of E-cadherin-p120 catenin interaction promotes the ubiquitination-dependent endocytosis of E-cadherin by binding of E-cadherin to Hakai, an E3-ubiquitin ligase, in a Src-dependent manner [89, 90].

The E-cadherin trafficking may also be compromised by post-translational modifications of E-cadherin which have been reported to promote apoptosis [91]. O-GlcNAc glycosylation of E-cadherin cytoplasmic domain and incomplete processing arrest E-cadherin transport late in the secretory pathway by interfering the binding of E-cadherin with type I gamma phosphatidylinositol phosphate kinase (PIPKIγ), a protein required for E-cadherin recruitment to the adhesion sites [92].

Regulation of E-cadherin-mediated cell- cell adhesion

Dynamic regulation of cadherin-mediated cell-cell adhesion is associated with diverse morphogenetic processes [93]. The crucial role of such dynamism is evident during embryonic development. In fact, E-cadherin is expressed from the very early stages of development, at the two-cell stage [94]. E-cadherin is the first adhesion molecule expressed in the mouse embryo, and is reported to be essential during morula compaction and blastocyst formation [95, 96]. E-cadherin null embryos failed to form a blastocyst cavity, which emphasizes the crucial role of this molecule in tissue morphogenesis and developments. Defects in cell junctional and cytoskeletal organization resulted in failure to maintain a polarized and compacted state and also failure to form a trophectoderm epithelium [97].

In adult tissues, epithelial cells also display dynamic behaviours, such as rearrangement, movement and shape changes implicated in developmental growth, cell renewal, cell migration, and wound healing [54, 59]. The remodelling of adherens junctions, which comprise cadherin adhesion molecules associated to cytoplasmic proteins, has major roles in controlling these behaviours. Endocytosis is one of the mechanisms that modulates the adherens junctions [98]. It has been shown that endocytic trafficking of E-cadherin controls assembly, disassembly and stabilization of adherens junctions [99]. In addition, E-cadherin-mediated junction is also regulated by several signal transduction pathways, which transduce changes across the membrane to alter the state of the cadherin adhesive bond [54]. However, E-cadherin is not only a target for signalling pathways that regulate adhesion, but may itself send signals that regulate basic cellular processes, such as migration, proliferation, apoptosis and cell differentiation [100, 101].

E-cadherin dysregulation in cancer

Disruption of cell-cell contacts and loss of cellular adhesion constitute a key step in tumour development and progression. In fact, E-cadherin is the main suppressor of epithelial tumour invasion, since E-cadherin impairment results in loss of cell adhesion and alterations of epithelial morphology, increased invasiveness and acquisition of metastatic potential, ultimately contributing to malignancy [102]. E-cadherin has also been implied in the process of epithelial-mesenchymal transition (EMT), a process described to be important in cancer metastasis [103].

The downregulation or inactivation of E-cadherin is particularly evident and significant in gastric cancer. The presence of non-cohesive cells with reduced intercellular adhesiveness is a defining feature of diffuse gastric carcinomas that exhibit an aberrant expression or complete loss of E-cadherin expression [104]. Several molecular mechanisms have been described to underlie E-cadherin dysfunction in cancer (Figure 2A), including: LOH of chromosome 16q21-22 [41, 42], mutations of the E-cadherin gene *CDH1* [40]; epigenetic silencing through promoter hypermethylation [43, 44]; transcriptional silencing that target *CDH1* promoter [45]; microRNAs (miRNAs) [105], and endocytosis along with proteolytic processing of E-cadherin [64]. The absence or aberrant E-cadherin expression has been identified in sporadic diffuse/mixed gastric cancer cases due to the presence of *CDH1* somatic mutations. Moreover, germline alterations of *CDH1* gene characterize the HDGC. These germline mutations of the E-cadherin gene were first described in 1998 and since then, 68 families carrying germline *CDH1* mutations have been identified worldwide including in Portugal [39, 106]. Furthermore, overexpression of E-cadherin transcriptional repressors has also been associated with E-cadherin dysfunction in several types of cancer. These transcription repressors include Snail, Slug, Twist and SIP/ZEB2 [107]. Another mechanism that leads to *CDH1* downregulation is the hypermethylation of its promoter that occurs in a large CpG island in the 5' proximal promoter region of *CDH1* [108]. In addition, miRNAs have also been reported as another level of E-cadherin regulation [109]. Moreover, alterations in the endocytic/recycling pathway of E-cadherin can also lead to its dysfunction in cancer [89, 110]. Loss or delocalization of p-120 catenin can lead to E-cadherin endocytosis affecting its stability at the cell membrane [111]. In addition the recycling of E-cadherin can be impaired by the ectodomain shedding of E-cadherin by metalloproteinases and other proteases [112].

Despite those above mentioned genetic/epigenetic mechanisms underlying E-cadherin dysfunction in cancer, there is still a high percentage (around 70%) of human epithelial invasive cancers, including human sporadic gastric cancer cases (mainly diffuse-type) displaying E-cadherin dysfunction that is not explained by any of the aforementioned mechanisms of E-cadherin genomic alterations [113]. This gap of knowledge constitutes a concern in the clinical practice and it is therefore of paramount important to address the mechanisms linking E-cadherin dysfunction to the tumorigenesis of gastric cancer. In this regard, we have been proposed the existence of another mechanism of E-cadherin (dys) regulation in cancer that operates at the posttranslational level of E-cadherin, the glycosylation (Figure 2C) [46].

Protein Glycosylation

The molecular mechanisms of neoplastic progression continue to be a fundamental focus of biomedical research. Although genetic and epigenetic changes have been reported to drive the progression of neoplasia [114, 115], posttranslational glycosylation may exert an equally powerful effect on the outcome of neoplastic disease [116, 117]. In fact, analogous to genomics and proteomics, glycomics aims to define the structure and functional roles of glycans in complex biological systems [118, 119]. The mammalian glycome repertoire- the spectrum of all glycans structures- is estimated to be 10-10^4 times larger than the proteome and far more complex than the genome and proteome [120-122].

Glycosylation consists in the covalent attachment of a carbohydrate to proteins and lipids producing different families of glycoconjugates [117] (Figure 3). Glycoprotein is a glycoconjugate in which a protein carries one or more glycans linked to a polypeptide backbone, usually via N- or O- linkage. N-linked glycans are attached to asparagine (Asn) residue of proteins in the consensus peptide sequence Asn- X- Ser/Thr, where X is any amino acid except proline [118]. O-glycans, particularly found on secreted or membrane-bound mucins, consist of O-linked glycan attached to serine (Ser) or threonine (Thr) residue which can be further extended resulting in different types of O-glycans structures [118, 123].

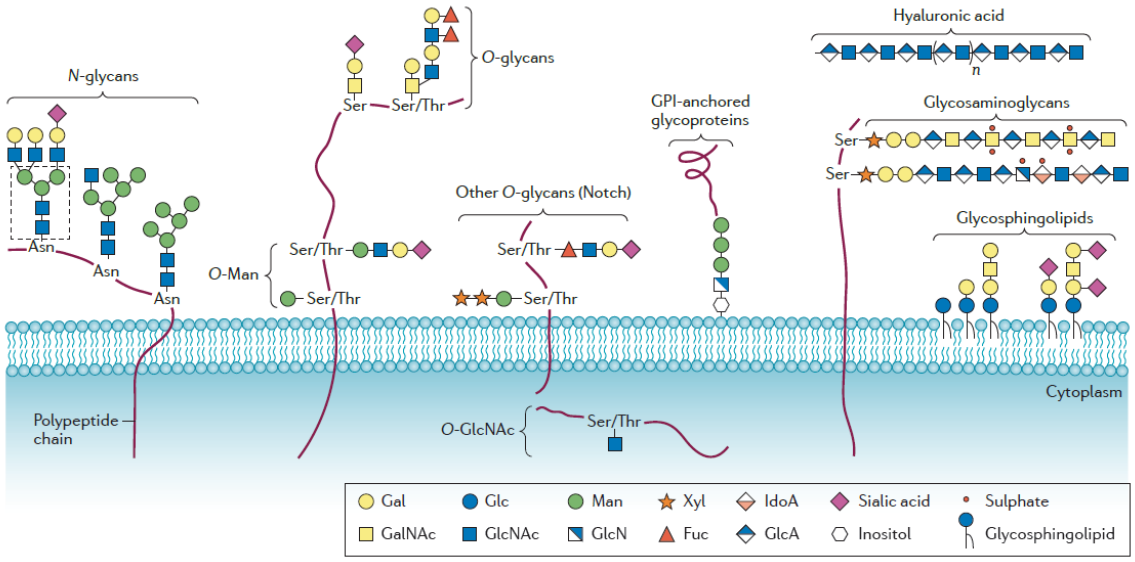

Figure 3 - Common classes of glycoconjugates in mammalian cells. Adapted from [117].

Protein glycosylation is not a random process but rather a non-template driven process [118], and characterized by the microheterogeneity phenomenon in which any single specific site on a protein may be occupied by a relatively limited number of glycan structures. The extent of microheterogeneity can vary from one glycosylation site to another, from glycoprotein to glycoprotein, and from cell to cell type. Several factors may affect glycan heterogeneity dictating the type of cell surface glycans present on a given glycoprotein: expression and localization of glycosyltransferases in the ER /Golgi complex, the ratio of their activities, their accessibility to substrate, the nucleotide sugar metabolism, as well as the Golgi pH [116, 117]. Such structural variation of glycans precludes the precise prediction of glycans structures in a given cell type.

Owing the complexity and dynamic nature of glycans, their biological roles span from nascent protein folding and intracellular trafficking to roles in molecular and cellular homeostasis [124], cell adhesion [125-127], cell-matrix [125] and host-pathogen interactions [47], immune modulation [128, 129], endocytosis, and signal transduction [124]. Therefore, minor alterations in glycan structure can significantly impact the structure and functions of glycoproteins by changing their conformation, stability, turnover, oligomerization, cell surface resident time, among other biological functions [117, 130].

N-glycosylation

The asparagine (N)-linked protein glycosylation occurs in all three domains of life: Bacteria, Archae and Eukarya [131]. In eukaryotic cells, about 90% of glycoproteins are likely to carry N-linked glycans with an average of 1,9 N-linked glycans per polypeptide chain [132]. The pathway initiates at the ER membrane where the glycan $Glc_3Man_9GlcNAc_2$ is synthesized and covalently coupled to the polypeptide backbone, and subjected to further trimming [133]. Here, the glycans have a common role in promoting protein folding, quality control and some sorting events [134]. Once the glycoproteins have folded and oligomerized correctly, they move to the Golgi complex, where N-glycans are extensively modified acquiring a diverse spectrum of structures and novel functions [118].

The **assembly of the core oligosaccharide** is performed by a series of glycosyltransferases that are encoded by asparagine linked glycosylation (*ALG*) genes (Figure 4). The three carbohydrate building blocks of the core oligosaccharide substrate (N-acetylglucosamine- GlcNAc, mannose- Man, and glucose- Glc residues) emerge from the primary metabolism and enter the pathway as nucleotide activated sugars [118].

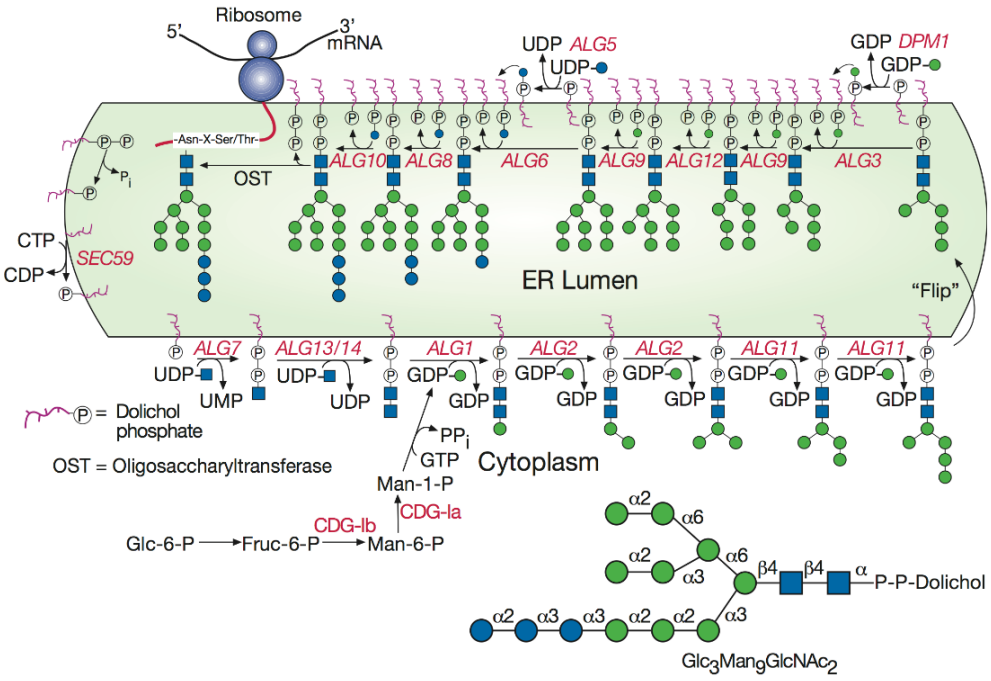

Figure 4 - Schematic representation of the N-linked oligosaccharide assembly. Adapted from [118].

The process starts on the cytoplasmic surface of the ER membrane by the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to the lipid-like precursor dolichol phosphate (Dol-P), forming GlcNAc-P-P-Dol. A second GlcNAc and five Man residues are subsequently transferred in a stepwise manner from UDP-GlcNAc and GDP-Man, respectively. The $Man_5GlcNAc_2$-P-P-Dol is then translocated to the ER luminal side by a bi-directional flippase [133]. Further addition of four Man residues from Dol-P-Man and three Glc residues donated by Dol-P-Glc to the partially synthesized N-glycans is carried out in a stepwise manner. Dol-P-Man and Dol-P-Glc donors are formed on the cytoplasmic face of the ER from GDP-Man and UDP-Glc through transfer of the respective sugar to Dol-P and "flipped" across the ER bilayer to the luminal face. At this point, the specific 14-residue oligosaccharide consisting of $Glc_3Man_9GlcNAc_2$ is completely synthesized to be further en-bloc transferred to the side-chain amide of Asn residues specified by the consensus sequence Asn-X-Ser/Thr site (where X is any amino acid except proline). This en bloc transfer is catalysed by the oligosaccharyltransferase (OST), a multisubunit protein complex associated with the translocon complex. OST binds to the membrane-anchored Dol-P-P-oligosaccharide and transfer the glycan to nascent protein by cleavage of GlcNAc-P bound, releasing Dol-P-P in the process [133]. The transfer of N-glycan to the innert side chain of Asn residues requires the formation of a loop so that the hydroxyl groups of Ser/Thr can contact the side-chain amide of Asn and render it more nucleophilic. The proline residue prevents the formation of such loop [135].

The hydrophilic carbohydrates attached to the protein alter the biophysical properties of the newly synthesized polypeptide increasing stability, solubility, and resistance to proteases [136-138]. In addition, the defined structures of the N-glycans serve as sorting signals creating a series of checkpoints to reflect the folding status of the glycoprotein [139]. Inhibition of glycosylation causes improper and incomplete folding of polypeptides and consequently failure to reach the native conformation. In this situation, these polypeptides are retained in the ER and targeted for degradation [140].

The **N-glycan processing** initiates immediately after the covalent attachment of the core glycan $Glc_3Man_9GlcNAc_2$ to the protein in the lumen of the ER (Figure 5). Terminal glucose residue is trimmed by α-glucosidase I generating the $Glc_2Man_9GlcNAc_2$ N-glycan which can associate with malectin. The possible functions of malectin include recruitment of α-glucosidase II for further processing and preventing aggregation of nascent polypeptides during the early synthesis [141].

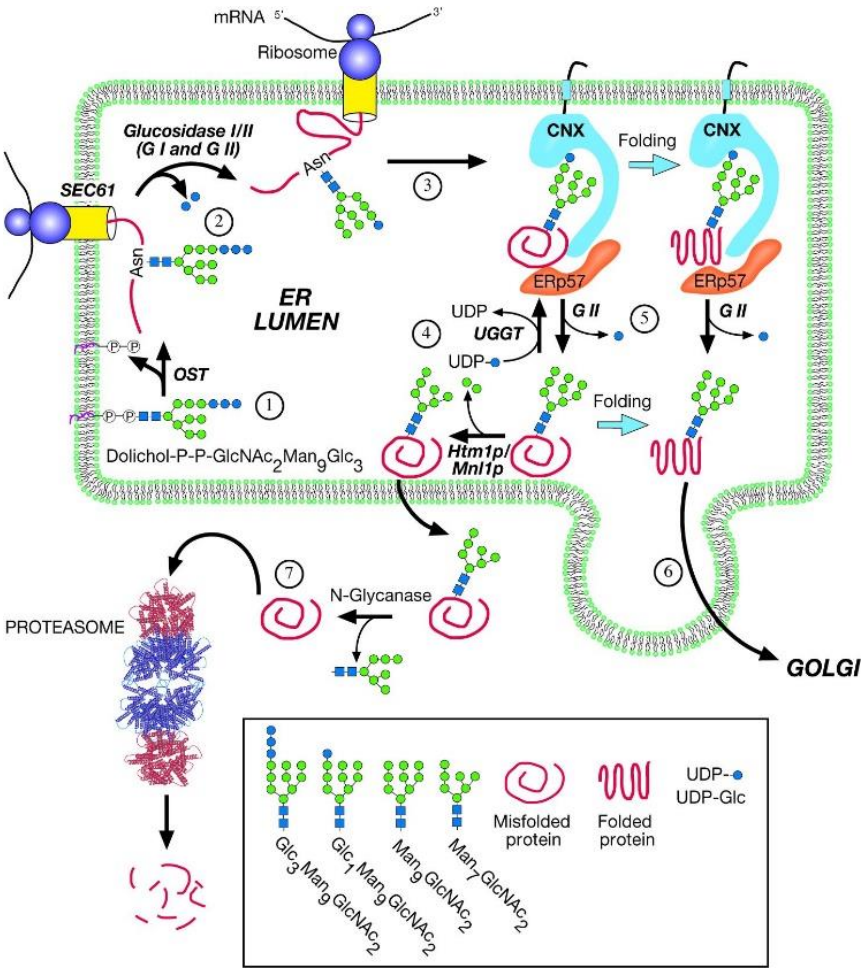

Figure 5 - Schematic representation of quality control of glycoprotein folding. Adapted from [118].

The next glucose residue is then removed by α-glucosidases II generating the monoglucosylated intermediate $Glc_1Man_9GlcNAc_2$, the ligand of the ER-resident lectin chaperones calreticulin (CRT) and calnexin (CNX), members of the protein quality control. Both proteins, in conjugation with ERp57, an ER resident oxidoreductase, function as the major chaperone complex in the CNX/CRT complex. The binding of these non-stable glycoprotein intermediates with CNX/CRT/ERp57 complex assists the proper protein folding and also prevent the protein aggregation [140-142].

Removal of the last glucose residue by glucosidase II causes the glycoprotein release from CNX/CRT cycle. At this point, properly folded proteins are packaged in COPII-coated vesicles and transferred to the Golgi. However, if the protein remained incompletely folded, the enzyme called UDP-Glc: glycoprotein glucosyltransferase (UGGT) catalyses the re-glucosylation generating a monoglucosylated N-glycan on the glycoprotein and consequently promoting the re-association with CNX/CRT complex. The cycle is repeated until the protein reached its native conformation. However, if proper folding of the protein still cannot be achieved, the unfolded glycoprotein further undergoes extensive mannose trimming which removes three to four mannose residues, generating $Man_{5-6}GlcNAc_2$.This intermediate will be then targeted to the ER- associated degradation (ERAD) pathway [139, 142].

After processing and trimming in the ER, the folded glycoproteins which arrive in the cis-Golgi are of the high mannose type containing usually eight or nine Man residues [143]. These high mannose-type N-glycans may remain unchanged during passage through the Golgi and be present on cell surface or secreted glycoproteins. However, further mannose trimming may occur in the cis compartment of the Golgi until to generate $Man_5GlcNAc_2Asn$, a key intermediate in the pathway to hybrid and complex N-glycans [118] (Figure 6).

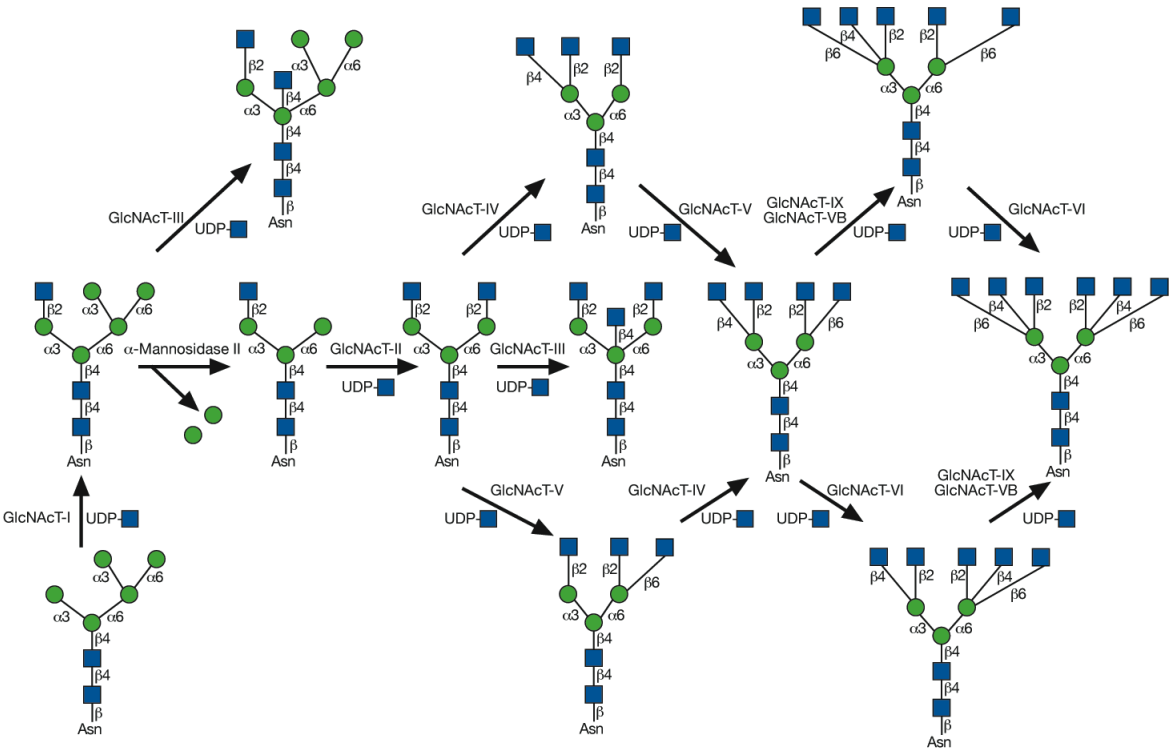

Figure 6 - Branching of complex N-glycans. Adapted from [118].

The Golgi membrane is covered with a spectrum of glycosyltransferases, glycosidases, and nucleotide sugar transporters that function together in a generally ordered manner from the cis-Golgi to the trans-Golgi network (TGN). Each Golgi glycosyltransferase transfers a sugar to a specific acceptor generated by preceding glycosyltransferases. Thus, Golgi glycosyltransferases must be appropriately localized in the cis-, medial-, trans- Golgi, or the TGN [143].

A set of α-mannosidases I in the cis-Golgi compartment removes mannose residues to generate the $Man_5GlcNAc_2$-Asn intermediate. After mannose trimming, N-acetylglucosaminyltransferase I (GlcNAcT-I or GnT-I), localized to the medial Golgi, catalyses the addition of the first GlcNAc residue to $Man_5GlcNAc_2$-Asn. This biosynthetic step is crucial for the conversion of high mannose to hybrid or complex-type N-glycans structures. Hybrid-type N-glycans keep the five mannose residues intact and extend the arm containing GlcNAc by addition of galactose (Gal), sialic acid (Neu5Ac) or other sugars. In complex-type N-glycans, the two outer mannose residues from $GlcNAcMan_5GlcNA_2$-Asn are removed by action of α-mannosidase II (another resident of the medial-Golgi) to form $GlcNAcMan_3GlcNAc_2$-Asn [118, 143].

Afterwards a second GlcNAc residue is added to mannose α1-6 in the core by the action of N-acetylglucosaminyltransferase II (GlcNAcT-II or GnT-II) to yield the precursor for all bi-antennary complex N-glycans. Further branching may occurs: N-acetylglucosaminyltransferase IV (GlcNAcT-IV or GnT-IV) adds a GlcNAc residue to the Manα1-3 arm via β4 linkage initiating the synthesis of tri-antennary structures. Another enzyme N-acetylglucosaminyltransferase V (GlcNAcT-V or GnT-V) catalyses the transfer of GlcNAcβ1,6 branch to the Manα1-6 arms of bi-and tri- antennary substrates to form tri- and tetra-antennary N-glycans respectively. Then, N-acetylglucosaminyltransferase VI (GlcNAcT-VI or GnT-VI) may add the final branch to N-glycans to generate petra-antennary structures [118, 143].

Hybrid and complex N-glycans may carry a “bisecting” GlcNAc residue linked to the internal β-Man residue of the core by N-acetylglucosaminyltransferase III (GlcNAcT-III or GnT-III). The presence of a bisecting GlcNAc inhibits many of the otherwise possible elongation and branching reactions [118].

During subsequent terminal glycosylation, further sugar additions may occur in the trans-Golgi: 1) fucose (Fuc) residue addition to the GlcNAc residue adjacent to Asn (after GlcNAcT-I action); 2) elongation of branch GlcNAc residues of N-glycans (e.g. poly-N-acetyllactosamine or polyLacNAc); 3) and “capping” and “decoration” of elongated branches (addition of Neu5Ac, Fuc, Gal, N-acetylgalactosamine- GalNAc) [118].

O-Mannosylation

Protein O-mannosylation is a posttranslational process that is initiated at the ER by the covalent attachment of mannose structures to Ser or Thr residues of secretory and/or membrane proteins catalysed by the homologous protein O-mannosyltransferase 1 (POMT1) and 2 (POMT2). These O-mannose core structures may be further extended via different linkages originating distinct extended O-mannosylated structures [144]. The O-mannosylation in mammalian proteins has been demonstrated to play crucial roles in several biological mechanisms such as infections, cell adhesion, neuronal development [145, 146], and in cellular interactions-based pathologies, including congenital muscular dystrophies (CMD) [147-150] and cancer metastasis [151-153].

In mammals, O-linked glycans initiated by mannose attachment were first detected in a proteoglycan-enriched fraction of rat brain lysate, and were considered to be present on a limited number of glycoproteins from brain, nerve, and skeletal muscles [154]. In mammalian brain tissues, O-mannose glycans account for up to 30% of all O-linked glycans

to proteins [150, 155, 156] . Moreover, O-mannosyl glycans are the major modifications of secreted and cell wall proteins in yeast which makes the eukaryotic model yeast *Saccharomyces cerevisiae* as the best model to characterize O-mannosylation pathway and function of O-mannosyl glycans [144].

The most well characterized O-mannosylated mammalian protein is α- dystroglycan (α-DG), an integral glycoprotein of the dystrophin-glycoprotein complex [157, 158]. The α-DG links the extracellular matrix (ECM) to the actin cytoskeleton by interacting with ECM proteins in a glycosylation-dependent manner. Disruption of the O-mannosylation pathway causing the hypoglycosylation of α-DG results in the impairment of α-DG-mediated epithelial cell-basement membrane interaction, and underlies various forms of CMDs [159], as well as cancer metastasis [160]. Recent glycomics and glycoproteomic studies using mass spectrometry technology demonstrated a wide spectrum of known O-mannosylated proteins and their implications in cell biological functions and pathologies [161]. Several additional proteins were thus identified to be modified with O-mannosyl glycans, including CD24 [162], receptor tyrosine phosphatase β (RPTPβ) [163], neurofascin 186 [164], lecticans [165], cadherin's and plexins [161]. However, the specific structures and the biological roles of O-mannosyl glycans in each proteins remain to be elucidated.

Beyond a structural role, O-mannosylation has been reported to be essential in ER protein quality control [166-170]. ER- stress situations trigger increased levels of protein O-mannosyltransferases (PMTs) by the unfolded protein response (UPR) [171]. Proteins which normally are not carriers of O-mannosyl glycans but failed to acquire the proper folding, undergo O-mannosylation in order to be targeted for degradation via proteasome-dependent ERAD pathway [168-170].

Evidences suggest that a single polypeptide translocating into the ER can undergo O- and/or N-glycosylation processes, and therefore competition between PMTs and OST enzyme complexes for acceptor proteins substrates was reported [144, 172]. Ecker et al demonstrated that N-glycosylation of cell wall protein Ccw5 only occurs in pmt4 mutant suggesting that O-mannosylation precedes and can prevents N-glycosylation [173]. Indeed, PMTs have the potential to alter N-glycosylation site occupancy: 24% of the identified glycopeptides specific N-glycans acceptor sequon were only used in pmt mutants. Nevertheless, Harty et al reported an opposite situation: N-glycans may introduce conformational changes which prevent O-mannosylation of N-glycosylated precursor [170]. Taken together, the potential crosstalk between O-mannosylation and N-glycosylation and its relevance in cell biology remain to be clarified, being a subject addressed on this PhD thesis.

The **biosynthesis of O-mannosyl glycans** is initiated at the cytosolic side of ER, where membrane dolichol is phosphorylated by the dolichol kinase Sec59, originating Dol-P [174]. The enzyme GDP-Man: Dol-P mannosyltransferase (Dpm1) is then responsible for the transfer of mannose residue from GDP-Man to Dol-P, resulting Dol-P-Man, the only mannosyl donor in all eukaryotes [175, 176]. Dol-P-Man is then flipped from the cytosol to the luminal side of the ER [177]. In the ER lumen, protein O-mannosylation is initiated by the covalent attachment of mannose to Ser or Thr residues of secretory and membrane proteins [176] (Figure 7). The O-mannose core structures can be further extended via GlcNAcβ1-2Man, GlcNAcβ1-4Man, or GlcNAcβ1-6Man linkage [178-181]. Twenty-three different O-mannosylated structures have been characterized so far [182].

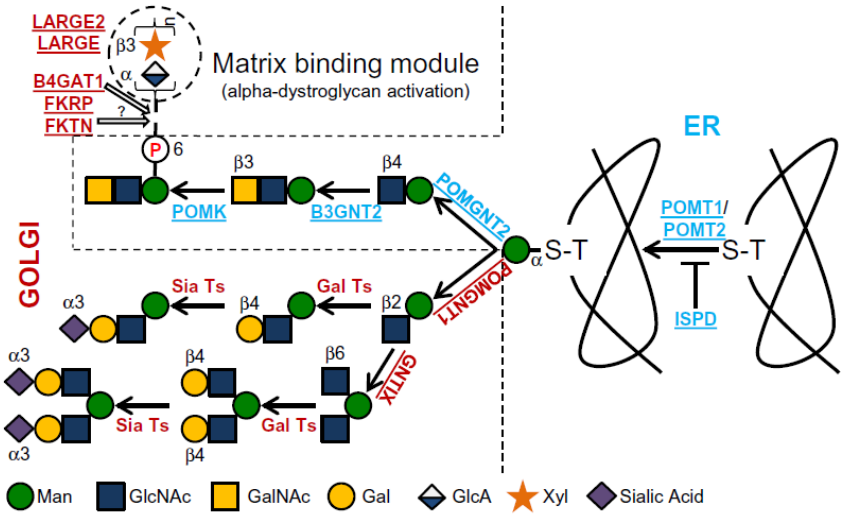

Figure 7 – Simplified O-mannosylation pathway. Adapted from [183].

The genes encoding PMTs, which initiates protein O-mannosylation, have been well characterized in the yeast *Saccharomyces cerevisiae* [144]. *Saccharomyces cerevisiae* comprises six PMTs grouped into three subfamilies, PMT1, PMT2, and PMT4 [184]. Two homologues, POMT1 and POMT2, have been identified in mammals [185] and classified as PMT4 and PMT2, respectively [186]. POMT1 and POMT2 are widely expressed in all mammalian tissues. These ER-resident proteins catalyse the initial step of O-mannosylation pathway- the transfer of mannose residue from Dol-P-Man to Ser and Thr residues of proteins in the secretory pathway via an α linkage [176, 187]. Formation of a POMT1 and POMT2 heterocomplex constitute a prerequisite for the proper O-mannosyltransferase activity in mammalian cells [187]. Knockout of POMT1 or POMT2 result in embryonic lethality and complete loss-of-function causing severe neuronal migration disorder, known as Walker-Warburg syndrome (WWD), the most severe phenotype of CMD [188, 189].

Concerning the **O-Mannose elongation**, one of the possible mannose-linkage structures existing in O-mannosyl glycans is GlcNAcβ1-2Man via protein O-Mannose β-1,2-N-acetylglucosaminyltransferase 1 (POMGnT1) activity [190]. This glycosyltransferase is expressed in a variety of mammalian tissues and is localized in the cis-Golgi [191]. After POMT1 and POMT2 actions, POMGnT1 catalyses the transfer of GlcNAc residue from UDP-GlcNAc into O-mannosyl-modified glycoprotein in a β2 linkage [192] (Figure 7). The muscle-eye-brain (MEB) disease is predominantly associated with POMGnT1 gene expression alterations [148, 193, 194].

An alternative elongation pathway implicates human protein O-mannose β-1,4-N-acetylglucosaminyltransferase 2 (POMGnT2) which is responsible for the transfer of GlcNAc from UDP-GlcNAc to O-mannose residue through β4-linkage [181]. *POMGNT2* knockout mice exhibited abnormal neuronal migration and die in the first day of birth [195]. This pathways involves further extension by β3GalNAc-T2 and subsequent phosphorylation of the mannose residue at addition C6 position by protein O-mannose kinase (POMK) enzyme, generating GalNAcβ1-3GlcNAcβ1-4(P-6)Man structure [181]. POMGnT2, β3GalNAc-T2 and POMK are ER-resident enzymes and POMK-mediated phosphorylation requires the GalNAcβ1-3GlcNAcβ1-4Man structure generated by prior action of POMGnT2 and β3GalNAc-T2 [181] (Figure 7).

Finally, the last alternative extension pathway involves the action of β1,6-N-acetylglucosaminyltransferase-IX/Vb (GnT-IX/ GnT-Vb). The O-mannose glycan GlcNAcβ1-2(GlcNAcβ1-6)Man structure has been identified in α-DG glycoprotein from mammalian brain [179, 180]. The GnT-Vb activity requires the previous action of POMGnT1 to generate the GlcNAcβ1-2Man structure [196] (Figure 7). Other enzymes have been identified to be involved in further extension of the three types of O-mannosyl glycans core structures [182]. Mutations in known and putative glycosyltransferases involved in the biosynthesis of O-mannosyl glycans have been associated with defects in proper glycosylation of α-DG giving rise to human diseases.

Glycosylation alterations associated with cancer

Malignant transformation is strongly associated with altered glycosylation patterns on the surface of cancer cells, being a key event in the process of tumour development and progression [197]. In fact, expression of aberrant glycans have been implicated during the various steps of tumour progression, including proliferation, invasion, metastasis and

angiogenesis [130]. Altered expression and activity of glycosyltransferases and chaperones and mislocalization of glycosyltransferases are the major factors that affect the protein glycosylation in tumour cells [117]. Owing to the wide range of glycans with different structures and levels of expression present on tumour cells, compared with their non-transformed counterparts, cancer-associated aberrant glycosylation has been considered a hallmark of neoplastic cells [117]. Cancer-specific glycan epitopes have been presented themselves as potential cancer biomarkers for diagnostic, prognostic purposes and also as appealing therapeutic targets [117].

The major changes in glycan structures during malignancy encompass alterations both in O- and N-glycans structures which may occur both at early and late stages of cancer progression and metastasis. The most-widely occurring cancer-associated changes in glycosylation are: increased sialylation, truncated O-glycan, fucosylation, and branching N-glycans [116, 117, 198]. During neoplastic progression, many tumours exhibited high levels of the sialylated antigens SLe^a, SLe^x [199, 200] and STn [201, 202]. Furthermore, core fucosylation have been reported to be related with cancer [203]. Upregulation of core fucosylation has been found in liver, ovarian and colon tumours tissues [203-205] and in the serum levels of hepatocellular carcinoma (HCC) and ovarian patients [205, 206].

N-linked glycans in cancer

The expression of complex β1,6-branched Asn-linked oligosaccharides structures have been directly associated with malignancy and metastatic potential of the neoplastic cells [207]. The β1,6-branched N-linked glycans results from the GnT-V activity, which is encoded by the mannoside acetylglucosaminyltransferase 5 (*MGAT5*) gene. During malignant transformation, *MGAT5* is upregulated by the activation of the RAS-RAF-MAPK signalling pathway, which leads to the expression of branching in Asn-linked oligosaccharides mediated by GnT-V and to the promotion of tumour development and potential [208]. However, it is important to note that the occurrence of β1,6 branching in asparagine-linked oligosaccharides is dependent on tissue-specific regulation of GnT-V activity [209].

The role of GnT-V-mediated branched N-glycans in the process of tumour development and progression has been highlighted through various mechanisms of action. In fact, the GnT-V-dependent N-glycan modifications have been reported to impair epithelial contact inhibition in immortalized lung epithelial cell line, being associated with significantly increased cellular motility and tumour formation in athymic mice [210]. Likewise,

overexpression of GnT-V enhances the invasiveness of glioma and colon cancer cell lines [211, 212], as well as in mouse mammary carcinoma cells [209]. Furthermore, early events in breast carcinogenesis were found to be regulated by GnT-V through modulation of her-2-mediated signalling pathways [213]. In addition, transfection of GnT-V in mouse mammary cancer cell lines resulted in a significant induction of tumour growth and metastasis [214]. Accordingly, *MGAT5*-deficient mice displays significant suppression of mammary tumour growth and metastasis [215].

GnT-V-modified N-glycans have also been reported to modulate the cell signalling function of surface receptors, and consequently be implicated in cancer invasion and metastasis [205]. In fact, the extension of β1,6 GlcNAc branched N-glycans with poly N-acetyllactosamine structures on the surface of glycoprotein receptors leads to their binding to the galectins, a family of carbohydrate-binding proteins, originating the lattices (galectin-glycan structures) [216]. In turn, the constitutive endocytosis of the glycoprotein receptors is prevented, which contributes ultimately to the increased cell motility and tumour formation [217, 218].

The β1,6 GlcNAc branched N-glycans structures also modulates the cell-ECM and cell-cell interactions [125]. The presence of these glycans on α5β1 and α3β1 integrins enhances the migration and invasion potential in human fibrosarcoma and melanoma cells, respectively [219, 220]. The branched N-glycans also regulates the biological functions of the adhesion molecule E-cadherin, thereby affecting cell-cell interaction (as described below). At the clinical point of view, the β1,6 GlcNAc branched N-glycans structures have been reported as a predictors of poor outcome of breast carcinoma [221, 222].

The bisecting GlcNAc structures, catalysed by GnT-III, have been shown to have an important role in a cancer context [223]. GnT-III activity has been reported to counteract the role of GnT-V in cancer, regulating cancer cell survival and progression. GnT-III, encoded by *MGAT3*, catalyses the addition of GlcNAc in a β1,4-linkage, suppressing additional processing and elongation of N-glycans such as the β1,6-GlcNAc-branched N-glycans. *MGAT3* transfection into mouse melanoma B16 cells with high metastatic potential resulted in a significant suppression of lung metastasis in mice due to a reduction of β1,6 GlcNAc branching glycans through competition with GnT-V [223].

The crucial role of GnT-III in the suppression of tumour metastasis results from the impairment of the cell-ECM interaction and promotion of the cell-cell adhesion [224]. The overexpression of GnT-III on α5 subunit reduces the α5β1 integrin binding to fibronectin, compromising the α5β1 integrin-mediated cell migration [225]. A similar impact was also

reported in MKN45 gastric cancer cells on α3β1 integrin-mediated cell spreading on fibronectin [226]. In contrast, the enhancement of cell-cell adhesion is acquired by the stabilizing effect that GnT-III confers to E-cadherin (as described below) [47].

E-cadherin Post-translational modifications. Implications in cancer

E-cadherin is a glycoprotein that is post-translationally modified by glycosylation. Glycans have been described to precisely regulate the tumour cell-cell-adhesion by directly interfering with E-cadherin biological functions. The human E-cadherin ectodomain comprises four potential N-glycosylation sites: two putative sites located at EC4 subdomain (Asn-554 and Asn-566) and the remaining two potential sites at the EC5 subdomain (Asn-618 and Asn-633) [46].

The pattern of E-cadherin N-glycosylation was described to occurs in cell-density dependent manner, being modified with complex type or hybrid/high mannose oligosaccharides, in sparse and dense cultures, respectively [227]. These dynamic changes in the E-cadherin N-glycosylation profile are biologically relevant, since sparse cells (mimicking proliferative conditions) are known to form immature adherens junctions while dense cultures (mimicking differentiated state) form mature adhesion belts [227, 228].

The N-glycan at Asn-633 was found to be required for E-cadherin expression, folding and trafficking [229]. E-cadherin unglycosylated at this specific site is arrested in ER as a misfolded protein being degraded via ERAD pathway [230]. In turn, N-glycosylation at Asn-554 and Asn-566 were described to be important for cell cycle progression [231]. Additionally, N-glycosylation have been reported to affect the adhesive function of E-cadherin through modifying the assembly and stability of adherens junctions. In fact, removal of N-glycans at Asn-554 and Asn-566 resulted in elevated tyrosine phosphorylation level of β-catenin and consequently a reduced β- and α-catenin expression at adherens junctions, thus impairing the adhesive function of E-cadherin [229].

Recent studies with the neural cadherin (N-cadherin) further suggest that initial EC1 cadherin-dependent *trans* adhesive bonds established between opposing cadherin monomers are followed by lateral cadherin interactions controlled by N-glycosylation [232]. Removal of β1,6-branched N-glycans at EC2 and EC3 resulted in an increased N-cadherin cis-dimerization capability [233].

Several reports highlighted that *DPAGT1* gene expression and protein N-glycosylation are coordinated with canonical Wnt signalling and E-cadherin- mediated cell-cell adhesion via positive and negative feedback loops [234]. In fact, canonical Wnt signalling pathway regulates *DPAGT1* transcription, which in turn affects N-glycosylation status of Wnt components to further promotes Wnt signalling- positive feedback [235]. Moreover, high expression levels of *DPAGT1* induce extensive N-glycosylation of E-cadherin with complex N-glycans, inhibiting E-cadherin mediated cell-cell adhesion [236] and impairing stability of adherens junctions [237]. In turn, reduced expression of DPAGT1 leads to hypoglycosylation of E-cadherin which culminate in depletion of β- and γ-catenin from promoter of target genes, and thereby in inhibition of canonical Wnt signalling [228]. An additional positive feedback loop was described between E-cadherin and GnT-III expression where E-cadherin mediated cell-cell adhesion upregulates GnT-III expression and its products (bisecting N-glycans) [238, 239], which in turn downregulate the tyrosine phosphorylation of β-catenin, contributing to suppression of invasion and metastasis [240].

Besides affecting the composition and maturity of adherens junctions, N-glycosylation indirectly controls the assembly of tight junctions (TJ). Actually, reduced E-cadherin N-glycosylation promotes recruitment of protein phosphatase 2A (PP2A) to adherens junctions [241]. As result, ZO-1 and other components of TJ become phosphorylated and participate in the assembly of TJ [241]. Furthermore, N-glycosylation impacts adherens junctions' interactions with the cytoskeleton. E-cadherin hypoglycosylation improves the interaction of E-cadherin-β-catenin complex with PP2A and dynein which in turn associate with microtubules [242]. In contrast, E-cadherin-γ-catenin complexes establish association with the actin cytoskeleton via α-catenin and vinculin [241, 242].

During malignant transformation, E-cadherin undergoes an extensive modification on its N-glycosylation profile [243]. We and others have been demonstrating that E-cadherin regulates the transcription of GnT-III that in turn through competition with GnT-V, can glycosylate E-cadherin and promote a membranous localization of E-cadherin [127], and improvement of the competence of adhesive complex [244]. The N-glycosylation mediated by GnT-III was also associated with inhibition of E-cadherin endocytosis [126] and a delayed turnover of E-cadherin at the cell surface. Furthermore, E-cadherin modified with bisecting GlcNAc N-glycans enhances intercellular adhesion by recruitment of catenins [126], and downregulates intracellular signalling pathways involved in cell motility, supporting its contribution to tumour suppression [231]. In addition, *MGAT3* glycogene expression and GnT-III-mediated E-cadherin glycosylation also contributes to an epithelial phenotype that

prevents the EMT process [245, 246]. Interestingly from the clinical point of view, it was demonstrated that E-cadherin from human gastric carcinoma is modified with GnT-V mediated branching N-glycans playing an important role on E-cadherin-mediated tumour invasion and progression [126].

GnT-V is known to be upregulated in gastric carcinoma [247], contributing to cancer cell invasion and metastases [215]. Gastric cancer cells overexpressing GnT-V display E-cadherin mislocalization [126]. Furthermore, GnT-V-mediated glycosylation on E-cadherin was shown to interfere with β-catenin and p120 catenin recruitment, disturbing the stability of adherens junctions and compromising cell-cell adhesion. Moreover, β1,6GlcNAc branched N-glycans on cadherins also affect downstream signalling pathways [248], contributing to increased cell migration and invasion [226]. Interestingly, patients with gastric carcinoma displaying loss of E-cadherin function (not explained at the genetic or structural level) exhibit an increase in β1,6GlcNAc-branched N-glycans on E-cadherin [126].

Taken together, it is clear that E-cadherin suffers a profound alteration on its N-glycosylation profile that accompany malignant transformation. However, the specific role of each N-glycans (structure-function relationship) on E-cadherin function in cancer remained to be clearly elucidated and will be addressed in this PhD thesis.

E-cadherin can also undergoes to increased fucosylation in cancer context. Some reports regarding E-cadherin core fucosylation was shown to have a negative effect on the cell-cell adhesion [249], suggesting that core fucosylated E-cadherin can serve as a promising prognostic marker for lung cancer patients [250]. However, other study reported that transfection of α1,6-fucosyltransferase (FUT8) improves binding of E-cadherin to β-catenin, through reduction of tyrosine 654 phosphorylation β-catenin and its transcriptional activity [251].

Recently, E-cadherin was identified as a major target for O-mannosylation [161, 252]. Lommel et al. demonstrated that O-mannosylation of E-cadherin is required for the morula to blastocyst transition before implantation [252]. Interestingly, O-mannosylation has shown to be crucial for E-cadherin-mediated cell-cell adhesion in a normal context [252]. Absence of O-mannosylated structures at the EC2-5 domains led to a disruption of E-cadherin localization [161]. Nonetheless, unravelling the molecular role of O-mannosyl glycans on E-cadherin as well as their impact on the modulation of E-cadherin adhesive properties in cancer progression remains to be elucidated and will be assessed in this PhD thesis.

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Rational and Aims

E-cadherin is a classical cell-cell adhesion molecule playing pivotal roles in epithelial tissue morphogenesis. The functional inactivation or downregulation of E-cadherin is considered to be a hallmark of the epithelial carcinogenetic process, being closely associated with tumour cell invasion and metastases, ultimately contributing to malignancy. Taking into account that around 70% of human sporadic gastric cancer cases (mainly diffuse subtype) displayed E-cadherin dysfunction that is not explained by altered epi/genetic mechanisms, the modification of E-cadherin by glycosylation may constitute an alternative mechanism for E-cadherin regulation in cancer.

The specific role of each glycan structure in the dysregulation of E-cadherin functions in cancer remains to be elucidated. Moreover, the molecular role of the recently identified O-mannose glycans in the regulation of E-cadherin in cancer and its potential interplay with N-glycans is far from being determined.

The general aim of the present work is to unravel the E-cadherin “sugar code” in a gastric cancer context, and thus sorting out the structural frameworks and biological roles of E-cadherin glycans, envisioning potential clinical applications.

Specific aims

1. **To identify the E-cadherin-dependent signalling pathway involved in the regulation of GnT-III-mediated E-cadherin N-glycosylation**

The functional crosstalk between E-cadherin-mediated cell-cell adhesion and the bisecting GlcNAc N-glycans were reported to contribute to suppression of cancer invasion and metastasis. In fact, E-cadherin-mediated cell-cell adhesion upregulates GnT-III expression and its product (bisecting N-glycans), that promotes a membrane localization of E-cadherin and an improvement of the adhesive complex stability. However, the signalling pathways involved in this regulation, associated with tumour suppression, remains to be elucidated.

In chapter II, we aimed to assess the underlying signalling pathway (E-cadherin-dependent) involved in the regulation of the bisecting GlcNAc N-glycans in the process of tumour progression of epithelial cancer cells.

2. To determine the N-glycosylation site-specific occupancy of E-cadherin in a gastric cancer context and to disclose the biological role of each N-glycan on E-cadherin (structure- function relationship).

E-cadherin glycoprotein has four potential N-glycosylation sites that are potentially responsible for controlling its biological functions. In a gastric cancer context, E-cadherin undergoes different N-glycosylation patterns with implications in its adhesive functions. In cancer, E-cadherin is predominantly modified with β1,6 GlcNAc N-glycans catalysed by GnT-V activity which induced a mislocalization of E-cadherin into the cytoplasm, decreasing the stability of E-cadherin-catenin complex and impairing its adhesive properties. Therefore, the identification and characterization of the site-specific E-cadherin N-glycans occupancy, and its functional role on E-cadherin functions remain to be identified, being of utmost relevance for the clinic.

In chapter III, we intended to evaluate the N-glycosylation site-specific occupancy on E-cadherin in a gastric cancer context. Furthermore, we aimed to unravel the specific function of each E-cadherin N-glycan structure in the modulation of E-cadherin functions in cancer (structure-function relationship), and therefore to decipher the E-cadherin N-glycan sugar code envisioning potential clinical applications.

3. To evaluate the O-mannosylation profile of E-cadherin in a gastric cancer context and to disclose the molecular role of O-mannosyl glycans and their interplay with N-glycans in the regulation of E-cadherin biological functions in cancer.

E-cadherin was recently identified as a major target of O-mannosylation being required for the morula to blastocyst transition before implantation. Interestingly, O-mannosylation was shown to be important for E-cadherin-mediated cell-cell adhesion under physiological circumstance. Nonetheless, the E-cadherin O-mannosylation profile as well as the molecular role of E-cadherin O-mannosyl glycans in its adhesive properties and how this novel post-translational modification interplays with E-cadherin N-glycans in a cancer context remain to be elucidated.

In chapter IV, we aimed to disclose the role of O-mannosylation in gastric carcinoma, and to uncover the impact of E-cadherin O-mannosylation in the modulation of its biological functions. Furthermore, we intended to uncover the mechanistic interplay between O-mannosyl glycans and β1,6 GlcNAc N-glycans on E-cadherin glycoprotein. We also pretended to find out such interplay in human diffuse gastric cancer patients and different mice models and to infer about the relationship between protein O-mannosylation and GnT-V-mediated N-glycosylation processes.

Chapter II

Insulin/IGF-I Signalling Pathways Enhances Tumour Cell Invasion through Bisecting GlcNAc N-glycans Modulation. An Interplay with E-cadherin.

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Insulin/IGF-I Signaling Pathways Enhances Tumor Cell Invasion through Bisecting GlcNAc N-glycans Modulation. An Interplay with E-Cadherin

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Abstract

Changes in glycosylation are considered a hallmark of cancer, and one of the key targets of glycosylation modifications is E-cadherin. We and others have previously demonstrated that E-cadherin has a role in the regulation of bisecting GlcNAc *N*-glycans expression, remaining to be determined the E-cadherin-dependent signaling pathway involved in this *N*-glycans expression regulation. In this study, we analysed the impact of E-cadherin expression in the activation profile of receptor tyrosine kinases such as insulin receptor (IR) and IGF-I receptor (IGF-IR). We demonstrated that exogenous E-cadherin expression inhibits IR, IGF-IR and ERK 1/2 phosphorylation. Stimulation with insulin and IGF-I in MDA-MD-435 cancer cells overexpressing E-cadherin induces a decrease of bisecting GlcNAc *N*-glycans that was accompanied with alterations on E-cadherin cellular localization. Concomitantly, IR/IGF-IR signaling activation induced a mesenchymal-like phenotype of cancer cells together with an increased tumor cell invasion capability. Altogether, these results demonstrate an interplay between E-cadherin and IR/IGF-IR signaling as major networking players in the regulation of bisecting *N*-glycans expression, with important effects in the modulation of epithelial characteristics and tumor cell invasion. Here we provide new insights into the role that Insulin/IGF-I signaling play during cancer progression through glycosylation modifications.

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Introduction

The Insulin Growth Factor (IGF) system in mammals comprises a dynamic network of proteins including ligands (IGF-I and IGF-II) and at least four associated receptors. The insulin receptor (IR), IGF-I receptor (IGF-IR), and insulin receptor-related receptor (IRR) belong to the tyrosine-kinase superfamily [1].

Insulin/IGF-I signaling pathways play a crucial role during malignant transformation [2]. The activation of these pathways has been related with increased proliferation, survival, metastatic potential and angiogenesis [3]. Therefore, the Insulin/IGF-I signaling pathway has been considered an appealing therapeutic target in cancer [4]. In this context, it was demonstrated that tumor growth in human tumor xenograft models was significantly reduced by using antibodies that inhibit the Insulin/IGF-I signaling [5,6]. Moreover, daily treatment with OSI-906, a dual inhibitor of the IGF-I and insulin receptors, resulted in tumor growth inhibition in the NCI-H292 xenografts [7]. Furthermore, recent studies have point out the importance of the insulin/IGF-I signaling in the loss of epithelial features of carcinoma cells [8,9]. It was shown that IGF-I

increases invasive potential inducing TGF-β1-mediated Epithelial to Mesenchymal Transition (EMT) in MCF-7 breast cancer cells [8].

E-cadherin is a cell-cell adhesion molecule with pivotal roles in the suppresion of tumor cell invasion and metastasis, being also a key molecular player in the EMT process [10]. Dysfunction of E-cadherin is considered a major event of more than 70% of human invasive carcinomas. Several mechanisms have been recently proposed to underlie E-cadherin down-regulation or inactivation in cancer, such as post-translational modifications by *N*-glycosylation [11–15].

It has been our long last interest to understand the role that glycans play during the carcinogenic process, particularly in the modulation and regulation of E-cadherin biological functions. In this context, we have previously demonstrated that E-cadherin functions can be specifically modulated by the presence of different oligosaccharide structures [15–17]. We have shown that during the acquisition of the malignant phenotype, E-cadherin suffered an increased modification with β1,6 GlcNAc branched *N*-glycans, catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V) [18,19], that was further demonstrated to induce a destabilization of E-cadherin-mediated cell-cell adhesion (adherens junction) with consequences to tumor progression [17]. Furthermore, it was shown the existence a bidirectional cross-talk between E-cadherin expression and the *N*-acetylglucosaminyltransferase III (GnT-III) [19,20]. The modification of E-cadherin with bisecting GlcNAc *N*-glycans, catalyzed by GnT-III, was shown to enhance cell–cell adhesion with increased stability of adherens junctions, which was associated with suppression of tumor progression [17,21]. In addition, the modification of the growth receptors with bisecting GlcNAc structures precludes their membranar stabilization and consequently their signaling activation, through the inhibition of further extension and elongation of the *N*-glycans with β1,6 GlcNAc branched structures [22,23] .

Taking into consideration the existence of a functional feedback loop between E-cadherin-mediated cell-cell adhesion and bisecting GlcNAc *N*-glycans in the suppression of cancer cell invasion, it remains to be identified which are the associated signaling pathways involved in this process. In this study, we aim to identify the E-cadherin-dependent signaling pathway involved in the regulation of *N*-glycosylation, particularly in the expression of bisecting GlcNAc *N*-glycans and their impact on the malignant phenotype of MDA-MB435 epithelial cancer cells. We herein demonstrate for the first time that on one hand E-cadherin expression induces a significant decrease in the phosphorylation levels of insulin and IGF-I receptors, which was accompanied with an increased modification of E-cadherin with bisecting GlcNAc structures, and a consequent suppression of tumor cell invasion. On the other hand, the activation of the insulin and IGF-1 signaling pathways induces a significant decrease of the bisecting GlcNAc *N*-glycans in general, and specifically on E-cadherin molecule. Concomitantly, we also observed that activation of Insulin/IGF-I signaling pathways leads to an increased tumor cell invasion. Stimulation of cancer cells with insulin and IGF-I growth factors led to a significant upregulation of the fibronectin mesenchymal marker, and an alteration of E-cadherin and β-catenin cellular localization.

Altogether, our results contribute to the identification of a novel molecular mechanism, involving insulin and IGF-I signaling in the modulation of bisecting GlcNAc *N*-glycans expression on E-cadherin, and their consequente impact in the modulation of the invasive phenotype.

Materials and Methods

Chemicals and antibodies

Mouse monoclonal anti-E-cadherin and anti-β-catenin antibodies were obtained from BD Biosciences. Rabbit monoclonal anti-p-IR/p-IGF-IR, anti-IR, anti-IGF-IR, anti-p-Akt(Ser473), anti-Akt, anti-p-ERK 1/2, anti-ERK1/2 and anti-fibronectin were obtained from Cell Signaling Technology. Mouse monoclonal anti-α-tubulin was purchased from Sigma. Rabbit polyconal IgG anti-actin and peroxidase-conjugated anti-rabbit and anti-mouse IgG were purchased from Santa Cruz Biotechnology. *Biotinylated Phaseolus vulgaris erythroagglutinin* (E-PHA) and *biotinylated Phaseolus vulgaris leucoagglutinin* (L-PHA) lectins were purchased from Vector Laboratories. IGF-I was obtained from Immunotools and Insulin from Sigma. Alexa Fluor 488 anti-mouse was obtained from Invitrogen.

Cell Culture and transfection

Human MDA-MB-435 cells (which endogenously lacks E-cadherin expression at both the mRNA and protein level) were previously stably transfected with the empty vector (MDA-MB-435+mock) or with wild-type E-cadherin (MDA-MB-435+E-cad) [24]. Cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, under a humidified atmosphere containing 5% CO2. Cell lines stably transfected were maintained under antibiotic selection. MKN45 gastric carcinoma cell line stably transfected with MGAT5 or with an empty vector (mock cells) [17] were kindly provided by Prof. Taniguchi. These cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (1000 µg/ml), under the selection of G418 (500 µg/ml) in 5% CO2.

Immunoprecipitation, Western blot and lectin blot analysis

Cell cultures were washed with phosphate-buffered saline (PBS) and then lysed in a solution containing 1% Triton X-100, 1% NP40, protease inhibitor cocktail (Roche 1 tablet/50 ml buffer) and phosphatase inhibitor cocktail (Sigma, 1:100 dilution). Total protein was quantified using a BCA protein assay kit (Pierce). For immunoprecipitation, equal amounts of total protein (750 µg) from each cell lysate were precleared with 25 µl of protein G-sepharose beads (Sigma) for 1–2 h. After centrifugation, the supernatant was incubated overnight with 5 µg of mouse monoclonal antibody against E-cadherin (BD Biosciences). After that, incubation with protein G-sepharose for 2 h was performed. Next, the beads were

washed three times with immunoprecipitation buffer and the immune complexes were released by boiling for 5 min at 95°C in Laemmli sampling. For Western blot, samples were subjected to 7.5% SDS–PAGE and the separated proteins were transferred to a nitrocellulose membrane. The blots were then probed with primary and pexoxidase-conjugated secondary antibodies or biotinylated lectins (Vector Laboratories). The proteins were visualized using an ECL chemiluminescence kit (GE Healthcare). Immunoreactive bands from lectin blots were then visualized using the Vector stain ABC kit (Vector Laboratories).

Analysis of mRNA expression by RT–PCR and real-time PCR

Total RNA from MDA-MB435+mock and MDA-MB435+E-cad cells were extracted with Tri-Reagent (Sigma) according to the manufacturer's protocol. Yield and quality of RNA were determined spectrophotometrically. 1000 ng of total RNA were reverse transcribed using the Superscript III RNase H Reverse Transcriptase kit (Invitrogen) according to the manufacturer's instructions. Quantitative Real-Time-PCR (qRT-PCR) was carried out in triplicates using source RNA from 3 distinct biological replicas for the target genes *CDH1* (E-cadherin, Hs01023895_m1), *Ocln* (Occludin, Hs.PT.49.14927371), *CTNNB1* (β-catenin, Hs00355045_m1), *Vim* (Vimentin, Hs.PT.47.14705389), *CDH2* (N-cadherin, Hs.PT.49.15618412), FN *1* (Fibronectin, Hs.PT.47.1565512) and for the endogenous control *GAPDH* (GAPDH, Hs.PT.51.1940505). qRT-PCR analysis of mRNA expression was performed using TaqMan Gene Expression Assays (*CDH1*, *CTNNB1*, Applied Biosystems) or PrimeTime qPCR Assays (*Ocln*, *Vim*, *CDH2*, FN *1*, *GAPDH*, Integrated DNA Technologies). Data was analysed by the comparative 2(-ΔΔCT) method [25]. For all data comparisons, the Student's T-Test was used (two tailed, unequal variance).

Immunofluorescence

Cells were platted on six-well plates with coverslips. After 90% of confluence, cells were washed three times with PBS supplemented with 100 mM CaCl2 and 100 mM MgCl2 (PBS/CM), permeabilized with 0.1%Triton X-100, and blocked with BSA 3% (in PBS/CM) for one hour. For E-cadherin or β-catenin staining, cells were incubated with mouse anti-E-cadherin (BD Biosciences; 1:200 diluted in BSA 5%; one hour of incubation) or mouse anti-β-catenin (BD Biosciences; 1:100 diluted in BSA 5%; one hour of incubation) monoclonal antibody, respectively. After primary antibody incubation, the cover slips were washed in PBS and then Alexa Fluor 488 anti-mouse was used as secondary antibody (Invitrogen; 1:500 diluted in BSA 5%; one hour of incubation in the dark). Finally, the cover slips were washed in PBS and mounted on slides using Vectashield with DAPI (Vector Laboratories). Immunofluorescent images were obtained using a Zeiss Imager.Z1 AxioCam MRm (Carl Zeiss) and a TCS SP5 II (Leica) Laser Scanning Confocal microscope (Figure S4).

Phospho-receptor tyrosine kinase array

Human Phospho-RTK Array kit for 42 RTKs was performed for both MDA-MB-435+mock and MDA-MB-435+E-cad according manufacturer's protocol (R&D systems). The proteins were visualized using an ECL chemiluminescence kit (GE Healthcare).

Cell invasion assay

Cells (5 x 10^4) were seeded in the upper surface of transwell inserts with 8 μm pore size (Costar) coated with Matrigel for 24 h. After stimulation with insulin or IGF-I, the cells on the upper surface were removed and those from the lower surface were fixed with ethanol for 10 min, stained with DAPI and observed using an Axio Observer.Z1 microscope (Carl Zeiss). For quantification, three fields were counted for each assay.

Densitometry and Statistical analysis

The protein levels were quantified by densitometry using LabWorks 4.6 software (Bio-Rad). The measurements were obtained from sub-exposed photographic films after quimioluminescence reaction and the values were normalized to the amount of housekeeping (actin or tubulin). Student's T-Test and one-way ANOVA were performed with GraphPad Prism 4.02 software (GraphPad Software Inc.). We considered data from three independent experiments to be statistically significant at $P<0.05$. Graphic data are presented as the mean + SEM.

Results

Exogenous E-cadherin expression induces an epithelial-like phenotype in MDA-MB-435 cells

We used MDA-MB-435 cancer cell line, that endogenously lacks E-cadherin expression, as a model. Stable transfection with human full-length E-cadherin in MDA-MB-435 was previously performed [24]. In order to characterize the MDA-MB-435 mock and E-cadherin transfected cells, we performed a Western blot analysis using anti-Ecadherin antibody. We observed that MDA-MB-435+E-cad express E-cadherin, whereas in MDA-MB-435+mock we did not observed detectable levels of E-cadherin (Figure 1A). In addition, we have observed, by phase contrast microscopy, that expression of E-cadherin was associated with an epithelial-like phenotype whereas MDA-MB-435+mock cells exhibited a fibroblastoid-like appearance with presence of cytoplasmic protusions (Figure 1B). In order to confirm these phenotypical changes induced by E-cadherin transfection, we performed qRT-PCR to determine the expression levels of epithelial (occludin and β-catenin) and mesenchymal (vimentin, fibronectin and N-cadherin) markers at the mRNA level [26]. We observed that, the MDA-MB-435+E-cad cells exhibited a significant decreased expression of mesenchymal markers, such as fibronectin and N-cadherin, comparing with MDA-MB-435+mock. (Figure 1C). These results showed that, as expected, induced over-expression of E-cadherin inhibits the spindle shape morphology and induces an epithelial-like phenotype in MDA-MB-435 cells.

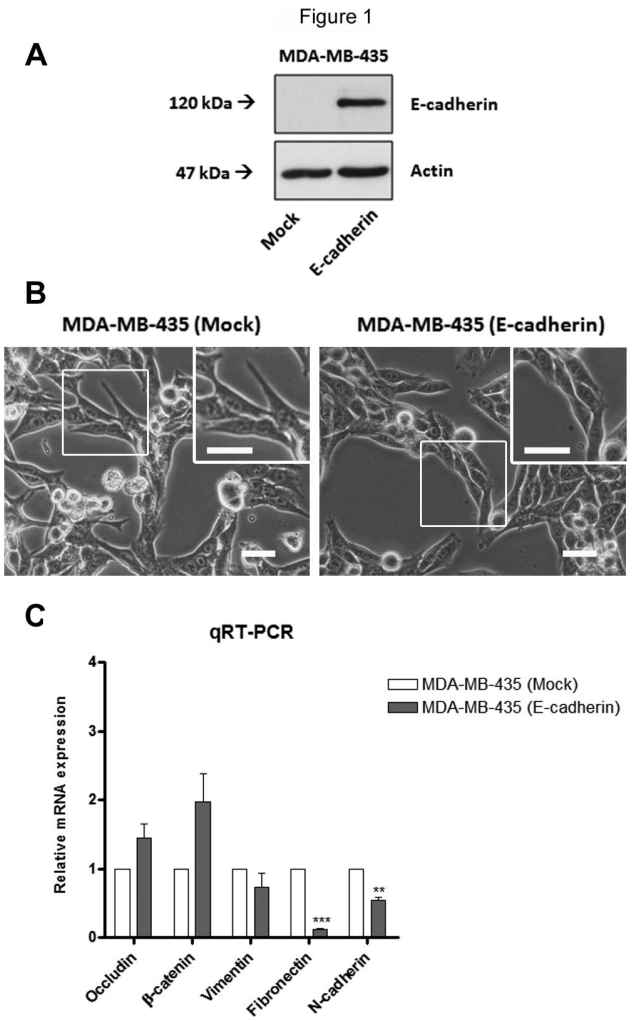

Figure 1. Effects of exogenous E-cadherin on the cell morfology and expression of epithelial and mesenchymal markers in MDA-MDA-435 cells. (A) Total cell lysates from MDA-MB-435+mock and MDA-MB-435+E-cad were obtained and analyzed by Western blot for E-cadherin. MDA-MB-435+mock cells do not show detectable levels of E-cadherin. Actin was used as a loading control. (B) The same amount of cells were seeded, and at the same time of culture phase contrast images show the cell morphology of MDA-MB-435+mock, which exhibit a mesenchymal-like phenotype, and MDA-MB-435+E-cad exhibit an epithelial-like phenotype. The inserts represent higher magnifications of the figure. Scale bar = 20 µm. (C) The bar graph shows the relative amount of E-cadherin, occludin, β-catenin, vimentin, fibonectin and N-cadherin mRNA levels by qRT-PCR. MDA-MB-435+E-cad cells exhibit a significant decreased expression of the mesenchymal markers fibronectin and N-cadherin. Values were normalized to the amount of mRNA in MDA-MB-435+mock. Error bars indicate the means + S.E.M. (n = 3). ** = P < 0.01, *** =P < 0.001, Student's t-test.

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Exogenous E-cadherin expression inhibits insulin/IGF-I signaling pathways in MDA-MB-435 cells

Activity of receptors tyrosine kinase (RTK) has been implicated in Epithelial-to-mesenchymal transition (EMT) program, which is turn is closely associated with tumor cell invasion and metastases [27]. We evaluated the impact of E-cadherin expression in the phosphorylation profile of RTK, by performing a phosphoproteome-array. Our results showed a marked decrease in the phosphorylation levels of insulin receptor and IGF-I receptor upon E-cadherin expression (Figure 2A and Figure S1). These results were further validated by Western blot analysis, showing a significant (P<0.01) decrease of phospho-IR(Tyr1150-51)/phospho-IGF-IR(Tyr1135-36) (Figure 2B and C). IR and IGF-IR can activate different downstream signaling pathways such as, Ras/Raf/MEK/ERK; PI3K/Akt and β-catenin [5,9]. In order to identify which signaling pathway is being modulated by exogenous E-cadherin expression, we analysed the protein expression levels of phospho-ERK1/2, total ERK1/2, phospho-Akt (Ser473), total Akt and β-catenin. The results demonstrated that E-cadherin expression led to a significant (P<0.01) decrease of ERK1/2 phosphorylation, and no significant changes of phospho-Akt (Ser473) were observed. No changes were observed for the expression of total Akt and ERK. In addition, we observed a slight increase, of the β-catenin protein expression levels upon induced E-cadherin over-expression (Figure 2B and C). These results suggest a relationship between E-cadherin expression and the modulation of IR/IGF-IR phosphorylation levels. In addition, the impact on the phosphorylation levels of ERK1/2 after E-cadherin expression further support that, in this cancer cell model, the IR/IGF-IR-mediated downstream pathway that is preferentially activated is the Ras/Raf/MEK/ERK.

Stimulation with insulin and IGF-I growth factors restore the phosphorylation of IR, IGF-IR and ERK 1/2, leading to cytoplasmic expression of E-cadherin and β-catenin

MDA-MB-435+E-cad were treated with growing concentrations of insulin and IGF-I growth factors. We first evaluated by Western blot whether these growth factors were able to restore phosphorylation levels of IR/IGF-IR and their downstream signaling pathway. The results showed that both, insulin and IGF-I, significantly (P<0.01) increased the phosphorylation levels of IR/IGF-IR (Figure 3A and 3C) and the phosphorylation levels of ERK 1/2 (P<0.01) in MDA-MB-435+E-cad cells (Figure 3B and 3C). Stimulation with insulin and IGF-I were not able to modulate the levels of phosphorylated Akt in our cell line model. Moreover, no significant changes were observed in E-cadherin and β-catenin proteins levels (Figure 3B and 3C). After stimulation with insulin or IGF-I, we have observed an impact in the cellular localization of E-cadherin and β-catenin. The results showed that upon insulin/IGF-I treatment there was a trend to an increased diffuse cytoplasmic expression of E-cadherin and β-catenin, as observed by immunofluorescence, together with some alterations in cellular morphology (appearance of

Figure 2

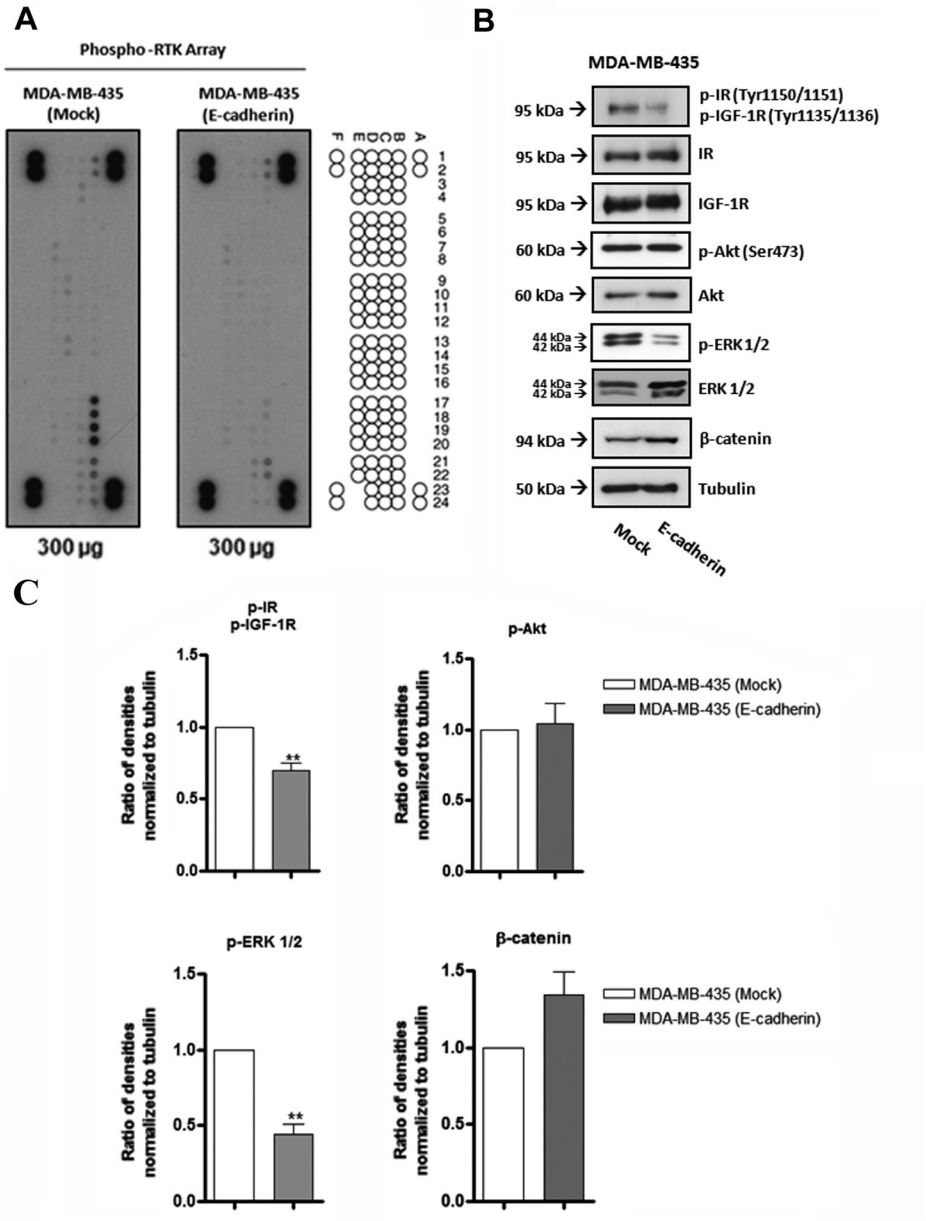

Figure 2. Effects of exogenous E-cadherin expression on the phosphoproteome profile of tyrosine kinase receptors and downstream proteins. (A) Total cell lysates from MDA-MB-435+mock and MDA-MB-435+E-cad were obtained and analyzed by Phospho-RTK array using 300 µg of proteins. The phosphor-RTK coordinates are shown on the right side of figure illustrating the localization of the spots containing immobilized antibodies on the nitrocellulose membrane. B17 and B18 represent the IR spots, whereas B19 and B20 represent IGF-IR spots, both receptors show decreased phosphorylation levels upon E-cadherin expression. (B) Total cell lysates from MDA-MB-435+mock and MDA-MB-435+E-cad were obtained and analyzed by Western blot for phospho-IR(Tyr1150-51)/phospho-IGF-IR(Tyr1135-36), IR, IGFR, Akt, phospho-Akt (Ser 473), ERK 1/2, phospho-ERK 1/2 and β-catenin. MDA-MB-435+E-cad cells show significant decreased levels of phospho-IR/phospho-IGF-IR and phospho-ERK1/2, comparing with mock cells. Tubulin was used as a loading control. The bar graphs show the relative amount of proteins levels normalized to tubulin. Error bars indicate the means + S.E.M. (n = 3). ** = $P < 0.01$, Student's t-test.

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cytoplasmic protusions, compatible with mesenchymal-like features) (Figure 4).

Activation of Insulin and IGF-I signaling pathways induces a decrease in the bisecting GlcNAc N-glycans expression in general and specifically on E-cadherin

Previous studies have demonstrated that E-cadherin induces an increased expression of bisecting GlcNAc *N*-glycans [19], which in turn is associated with an increased stability of E-cadherin-mediated cell-cell adhesion [17]. However, it remained to be determined the E-cadherin-dependent signaling pathway involved in this glycosylation modulation. Taking into consideration the inhibition of insulin/IGF-I signaling pathways induced by E-cadherin expression, we went further and assessed the impact of Insulin and IGF-I signaling pathways in the modulation of bisecting GlcNAc *N*-glycans expression, in MDA-MB-435 cancer cells. We first evaluated, by lectin blot analysis, the impact of insulin and IGF-I stimulation in the total expression levels of bisecting GlcNAc *N*-glycans [21,28]. We observed that both insulin (100 ng/mL) and IGF-I (50 ng/mL) stimulation of MDA-MB-435+E-cad cells induced a significant decrease ($P<0.01$) of the overall levels of bisecting GlcNAc *N*-glycans (Figure 5A). The levels of expresion of bisecting GlcNAc *N*-glycans in MDA-MB-435+Ecad cells stimulated with higher concentrations of insulin and IGF are similar to those expressed in mock cells. Furthermore, we evaluated the effect of this bisecting GlcNAc *N*-glycans modulation specifically on E-cadherin molecule. This was performed by immunoprecipitation of E-cadherin followed by lectin blot analysis using E-PHA lectin, that recognizes the bisecting GlcNAc structures. The results showed that activation of insulin and IGF-I signaling pathway led to a decreased modification of E-cadherin with bisecting GlcNAc structures (Figure 5B).

Stimulation of MDA-MB-435+E-cad cells with Insulin and IGF-I up-regulates mesenchymal markers and enhances tumor cell invasion

Since we observed a reduction of E-cadherin modification with bisecting GlcNAc *N*-glycans upon insulin and IGF-I treatment, which was accompanied with alterations in E-cadherin cellular localization and some alterations in cell morphology, we next assessed the impact of insulin and IGF-I signaling in cell differentiation and cellular function of MDA-MB-435 cells expressing E-cadherin. We evaluated the effect of insulin and IGF-I stimulation in the expression profile of EMT markers as well as in tumor cell invasion. Our results showed that both insulin and IGF-I stimulation induced a significant down-regulation of the mRNA levels of some epithelial markers (occludin and β-catenin) and an up-regulation of the mesenchymal marker fibronectin as evaluated at the mRNA levels (Figure 6A). Corroborating this result, stimulation with insulin and IGF-I also increased the fibronectin expression at the protein level, which has been closely associated with tumor cell invasion [27] (Figure 6B). E-cadherin, vimentin and N-cadherin did not undergo significant changes at the mRNA levels (Figure 6A). Interestingly, our results further showed that activation of insulin and IGF-I signaling, through growth factor stimulation, significantly enhanced ($P<0.01$) tumor cell invasion of MDA-MB-435+Ecad (Figure 7). Taken together, our results demonstrated that insulin and IGF-I signaling could in fact promote an invasive phenotype by changing the bisecting GlcNAc *N*-glycosylation profile of E-cadherin and consequently the expression pattern of epithelial/mesenchymal markers of cancer cells.

Discussion

Insulin/IGF-I signaling pathways have been widely implicated in the processs of tumor development and progression of different types of cancer being considered an important target with promising therapeutic aplications in cancer.

Aberrant glycosylation is considered as a key event in the process of tumor cell invasion and metastasis [29]. One of the most widely occurring glycosylation changes inducing malignancy is enhanced β1,6GlcNAc branching of *N*-linked structure, catalyzed by *N*-acetylglucosaminyltransferase V (GnT-V), which is counteracted by the synthesis of bisecting GlcNAc *N*-glycans catalyzed by *N*-acetylglucosaminyltransferase III (GnT-III) [28]. Increased bisecting GlcNAc *N*-glycans inhibits β1,6GlcNAc branching, leading to suppression of metastasis [20]. In this process, one of the target molecules is E-cadherin, in which the bisecting GlcNAc *N*-glycans is associated with enhanced cell-cell adhesion and increased stability of intercellular adherens-junctions, consequently contributing to suppression of tumor progression [15,17,21,30,31]. Previously, we and others have shown that the E-cadherin glycoprotein has a role in the regulation of bisecting GlcNAc *N*-glycans expression [19]. However, it remained to identify which signaling pathways (E-cadherin-dependent) were involved in this regulation of glycosylation, and associated with tumor supression. In the present study we aimed to assess the underlying signaling pathway (E-cadherin-dependent) involved in the regulation of the bisecting GlcNAc *N*-glycans in the process of tumor progression of epithelial cancer cells.

We have evaluated the impact of E-cadherin *de novo* expression in the activity of different receptors tyrosine kinase using an epithelial cancer cell model. Our results demonstrated that MDA-MB-435 cancer cells, lacking endogenous E-cadherin expression, exhibited a significant increased phosphorylation of IR/IGF-IR RTK showing also decreased levels of bisecting GlcNAc *N*-glycan structures. Upon exogenous overexpression of E-cadherin, there was a remarkable inhibition of the IR/IGF-IR phosphorylation suggesting the existence of an inhibitory effect of E-cadherin in the activity of Insulin and IGF-I signaling pathways. In fact, and in accordance with our observation, in hormone refractory PC-3 prostate cancer cells, IGF-I induced a decrease of E-cadherin expression levels [9]. However, in hepatocellular carcinoma cells the scenario observed seems to be the contrary, since the epithelial phenotype was strongly associated with expression of IGF-2 and IR as well as activation of IGF-1R and IR [32]. These conflicting observations suggest that the phosphorylation pattern of IR/IGF-IR appears to be tissue/cell-specific.

Figure 3

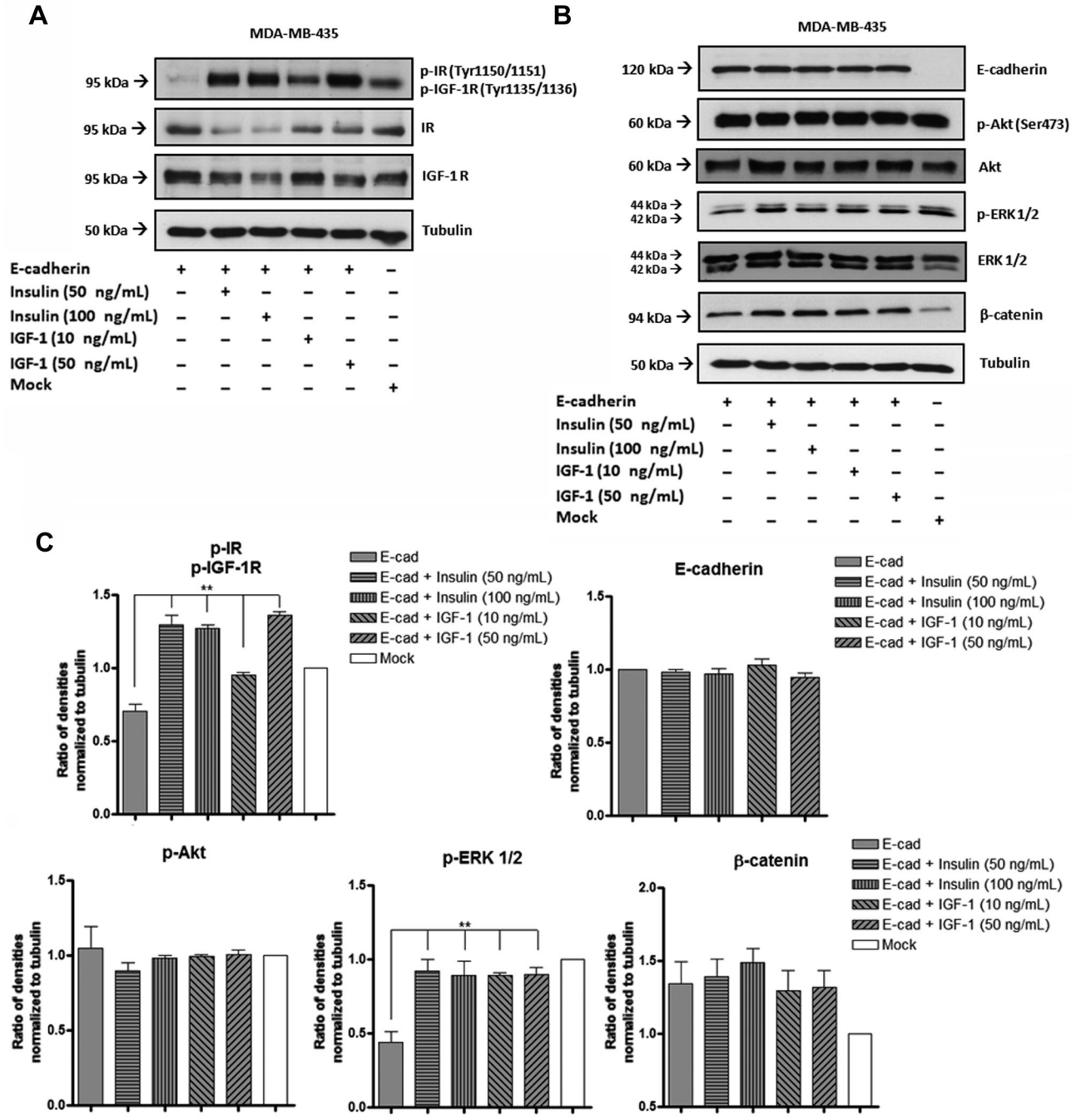

Figure 3. Effects of stimulation with insulin and IGF-I on the phosphorylation of tyrosine kinase receptors and downstream proteins. (A,B) Total cell lysates from MDA-MB-435+mock, MDA-MB-435+E-cad and MDA-MB-435+E-cad stimulated (24h) with insulin or IGF-1 were obtained and analyzed by Western blot for phospho-IR(Tyr1150-51)/phospho-IGF-IR(Tyr1135-36), IR, IGFR, Akt, phospho-Akt (Ser 473), ERK 1/2, phospho-ERK 1/2, β-catenin and E-cadherin. Increased phosphorylation levels of IR, IGF-IR, and ERK 1/2 were observed after stimulation with insulin or IGF-I. Tubulin was used as a loading control. No changes were observed for the total expression levels of Akt and ERK1/2. (C) The bar graphs show the relative amount of proteins levels normalized to tubulin. Error bars indicate the means + S.E.M. (n = 3). ** = $P < 0.01$, ANOVA t-test.

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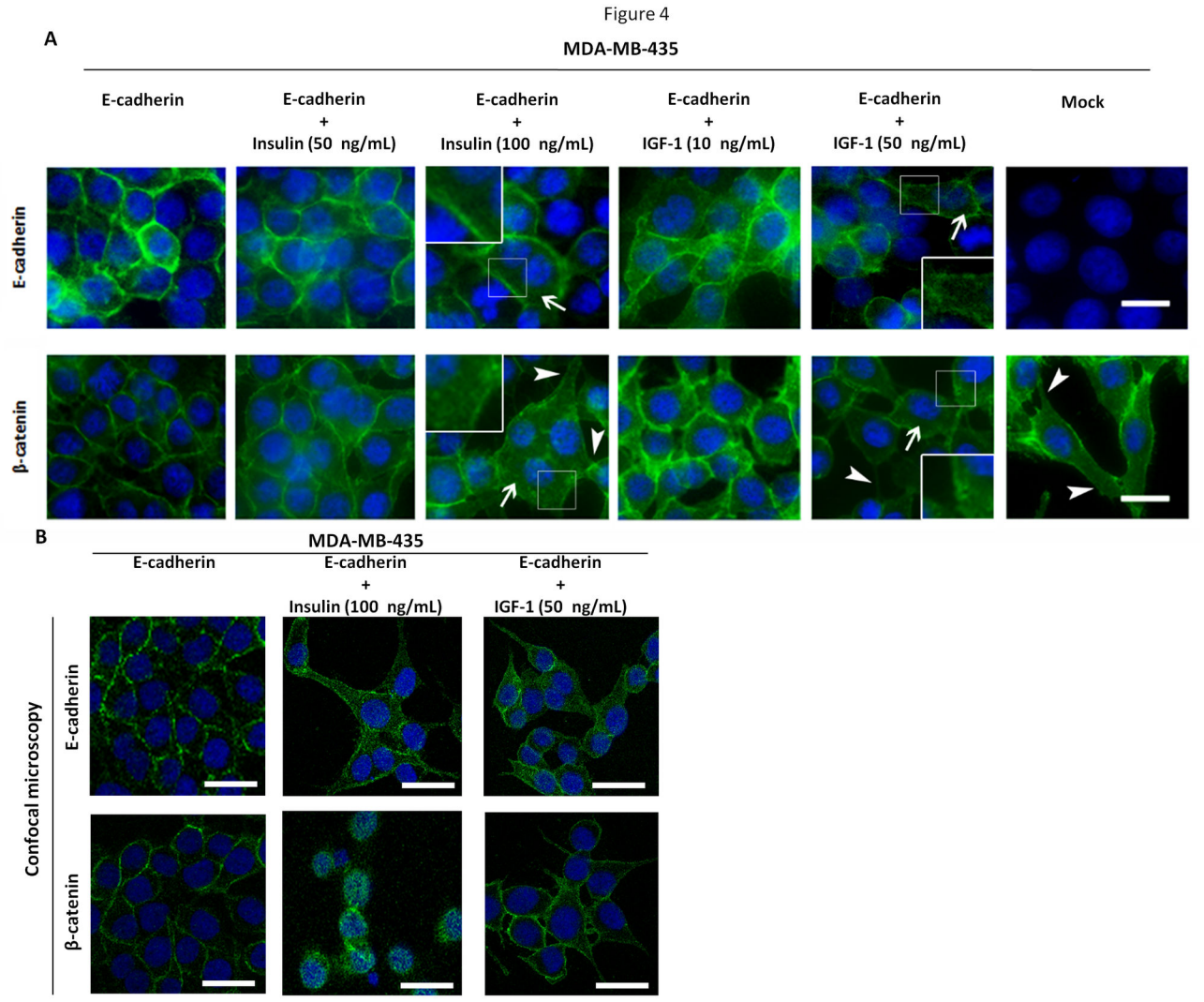

Figure 4. Subcellular localization of E-cadherin and β-catenin after stimulation with insulin and IGF-I. (A) Cell monolayers from MDA-MB-435+mock, MDA-MB-435+E-cad and MDA-MB-435+E-cad stimulated (24h) with insulin or IGF-1 were fixed and stained for E-cadherin, β-catenin and nucleus (DAPI). Diffuse cytoplasmic expression levels of β-catenin and E-cadherin are observed (arrows) after stimulation with insulin or IGF-I, together with observation of cytoplasmic protusions compatible with a fibroblastoid-like appearance (arrowheads). The representative images were obtained by fluorescence microscopy. White arrows and magnified images indicate cytoplasmic staining. Bar = 10 μm. (B) Confocal microscopy showing the cytoplasmic staining of E-cadherin and β-catenin after stimulation with insulin and IGF-I. Bar = 10 μm.

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Furthermore, our results showed that ERK 1/2 protein also undergoes a significant decrease in the phosphorylation levels upon E-cadherin expression, while no changes were observed in phospho-Akt (Ser473). These observations suggest that in MDA-MD-435 cancer cells, E-cadherin expression induces a downregulation of Ras/Raf/MEK/ERK downstream pathway through the significant decreased activity of the IR/IGF-IR signaling.

The activation of the IR/IGF-IR signaling and the downstream pathway Ras/Raf/MEK/ERK by insulin and IGF-I treatment led to a slight increase in the cytoplasmic expression of E-cadherin and β-catenin, that was accompanied with some alterations of cell morphology with observation of cytoplasmic extrusion compatible with an acquisition of a fibroblastoid-like appearance. However, this variation in cellular localization after stimulation with insulin or IGF-I appears to be not due to changes at E-cadherin and β-catenin protein levels (Figure 3). Curiously, after stimulation with Insulin or IGF-I, the mRNA levels of β-catenin underwent a significant reduction. This relationship between IR/IGF-IR signaling and β-catenin localization could be associated with a possible involvement of the activation of the WNT signaling pathway, as previoulsy

Figure 5

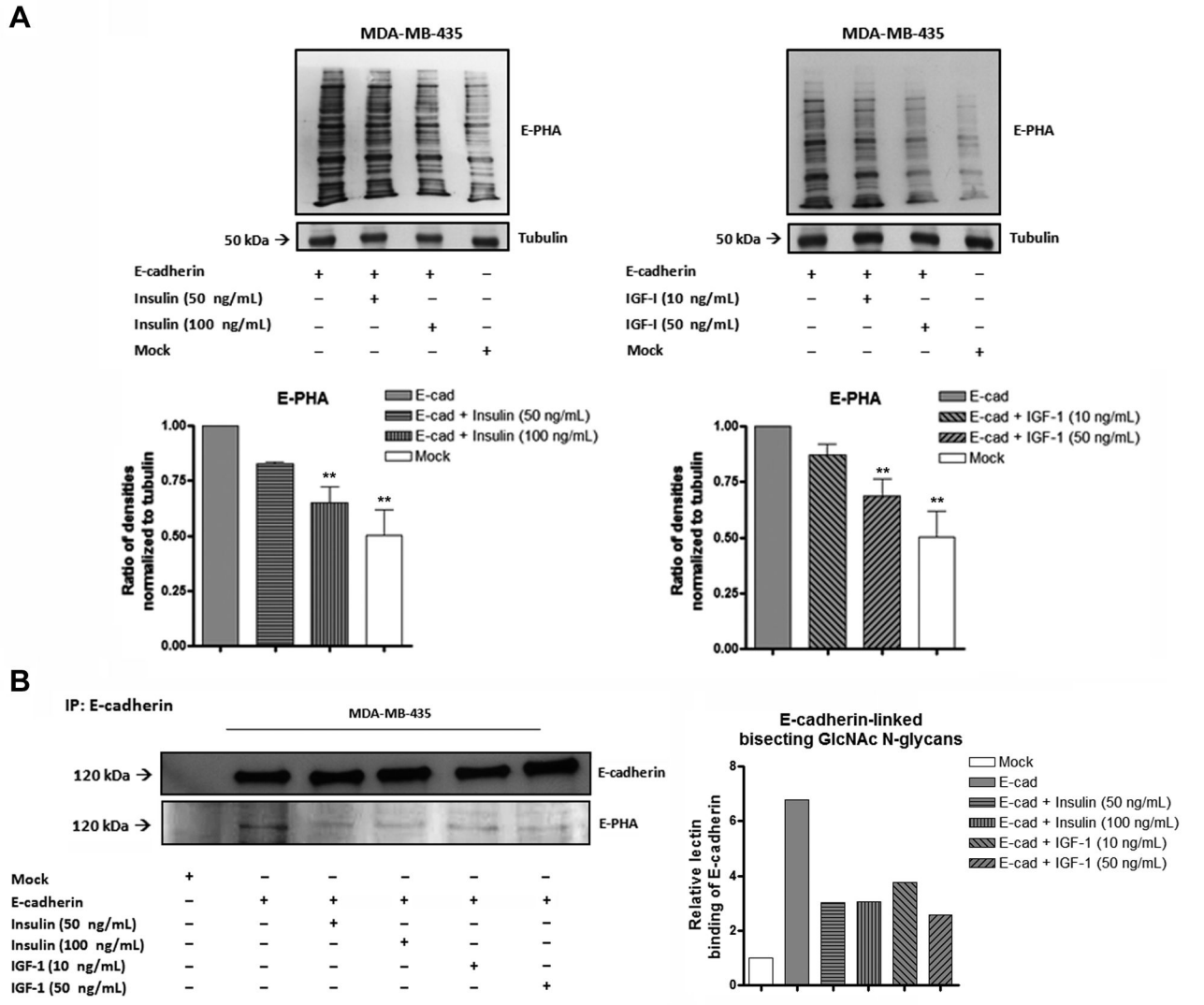

Figure 5. Effects of insulin and IGF-I stimulation on the expression levels of bisecting GlcNAc *N*-glycans, in general and specifically on E-cadherin. (A) Total cell lysates from MDA-MB-435+mock, MDA-MB-435+E-cad and MDA-MB-435+E-cad stimulated (24h) with insulin or IGF-1 were obtained and analyzed by Lectin blot for E-PHA. The bar graphs show the relative amount of bisecting GlcNAc *N*-glycans levels in the whole protein lysate. MDA-MB-435+E-cad cells stimulated with insulin (100 ng/mL) and IGF-I (50 ng/mL) showed a significant decrease of the overall levels of bisecting GlcNAc N-glycans. The values were normalized to tubulin. Error bars indicate the means + S.E.M. (n = 3). ** = $P < 0.01$ ANOVA test. (B) Total cell lysates from MDA-MB-435+mock, MDA-MB-435+E-cad and MDA-MB-435+E-cad stimulated (24h) with insulin or IGF-1 were obtained and immunoprecipitated using E-cadherin antibody. The immunoprecipitates were analyzed by Western blot for E-cadherin and Lectin blot for E-PHA. The bar graphs show the relative amount of E-cadherin-linked bisecting GlcNAc *N*-glycans levels. Activation of insulin and IGF-I signaling pathway led to a decreased modification of E-cadherin with bisecting GlcNAc N-glycan structures.

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described [33]. However, the mechanism underlying such modulation in the β-catenin expression need to be further investigated. Having identified IR/IGF-IR as one of the signaling pathways which activation is modulated by E-cadherin expression, we further evaluated the impact of this pathway in the regulation of glycosylation particularly the bisecting GlcNAc *N*-glycans, whose expression was described to be, in turn, regulated in an E-cadherin-dependent manner [19]. We have showed that Insulin and IGF-I stimulation induced a significant decrease of the overall levels of bisecting GlcNAc *N*-glycans expression. Furthermore, we demonstrated that this decrease through IR/IGF-IR signaling specifically

Figure 6

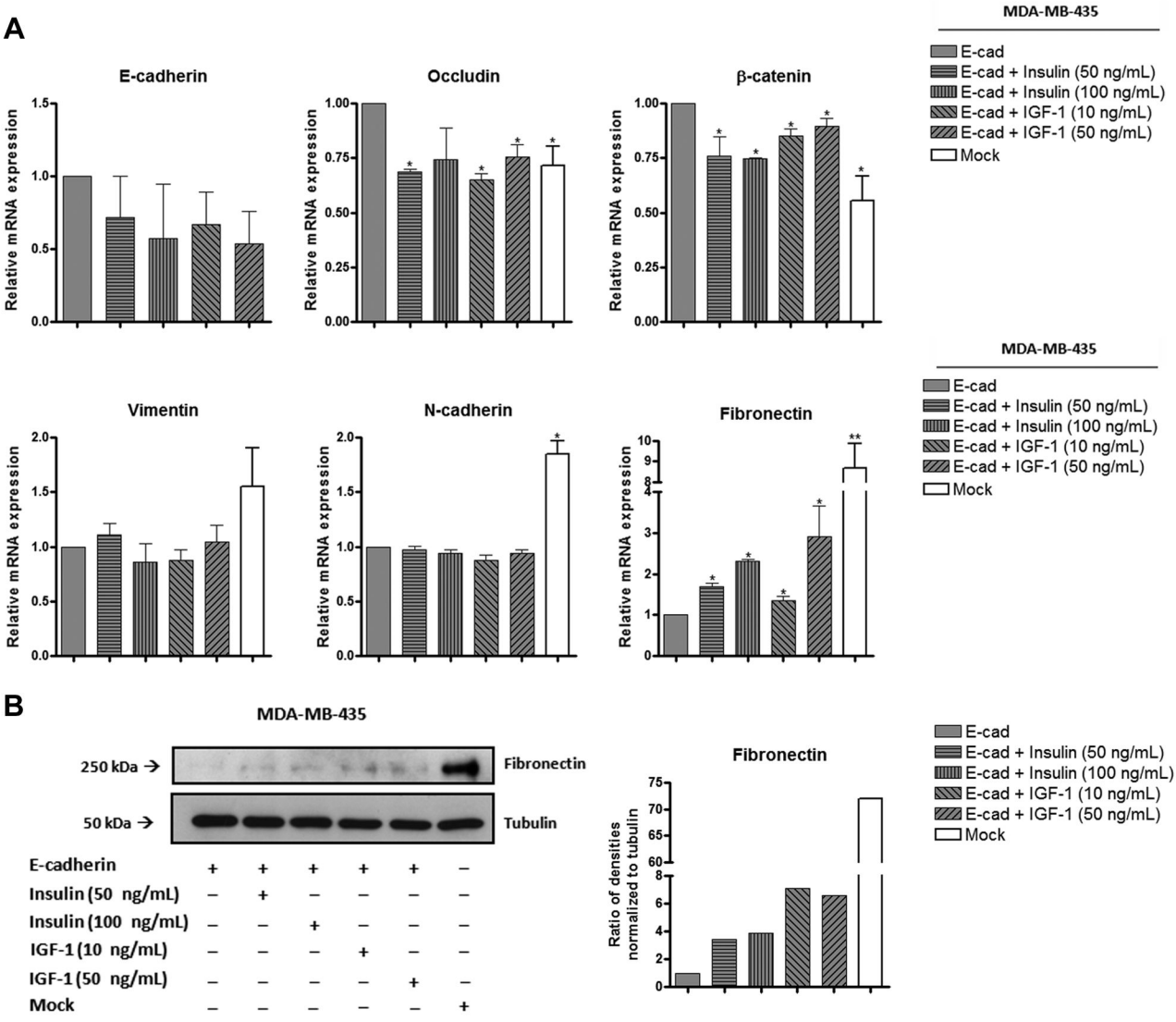

Figure 6. Effects of insulin and IGF-I stimulation on the mRNA expression levels of epithelial and mesenchymal markers. (A) The bar graphs show the relative amount of E-cadherin, occludin, β-catenin, vimentin, fibronectin and N-cadherin mRNA levels by qRT-PCR. Significant down-regulation of the mRNA levels of epithelial markers (occludin and β-catenin) and an up-regulation of the mesenchymal marker fibronectin were observed after insulin and IGF-I stimulation. Values were normalized to the amount of mRNA in MDA-MB-435+E-cad. Error bars indicate the means + S.E.M. (n = 3). * = $P < 0.05$, ** = $P < 0.01$, ANOVA test. **Effects of stimulation with insulin and IGF-I on the fibronectin protein levels**. (B) Total cell lysates from MDA-MB-435+E-cad,MDA-MB-435+E-cad stimulated (24h) with insulin or IGF-1, and MDA-MB-435+mock were obtained and analyzed by Western blot for fibronectin. Increased fibronectin expression levels are observed after stimulation with insulin or IGF-I. The bar graphs show the relative amount of fibronectin levels normalized to tubulin.

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targets E-cadherin molecule inducing a decreased modification of these bisecting GlcNAc *N*-glycans attached to E-cadherin glycoprotein. To the best of our knowledge, this is the first study showing such a relationship between IR/IGF-IR signaling and the modulation of bisecting GlcNAc *N*-glycans expression in general and specifically on E-cadherin. Interestingly, we further observed that IR/IGF-IR activation induced a significant increased tumor invasion capability of cancer cells. In addition, the signaling activation of IR/IGF-IR concomitantly induced modulation of expression of epithelial *versus* mesenchymal markers. We showed that IR/IFG-IR signaling activation induced an increased expression of the mesenchymal marker

Figure 7

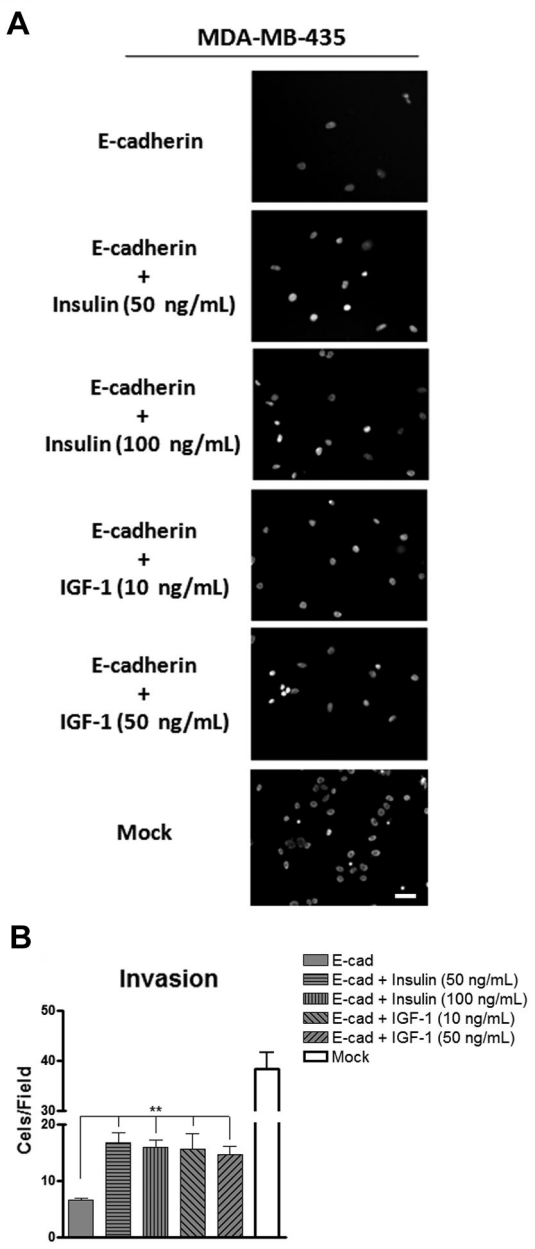

Figure 7. Effects of stimulation with insulin and IGF-I on cell invasion. Representative images of cell invasion through Matrigel using 8 mm pore of a polycarbonate membrane. Nuclei were stained with DAPI. We observed a significant increase of tumor cell invasion of MDA-MB-435+Ecad after stimulation with insulin or IGF-I. The bar graph shows the amount of cells/field. Error bars indicate the means + S.E.M. (n = 3). ** = P < 0.01 ANOVA test.

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fibronectin (both at protein and mRNA levels), together with a decreased expression of the epithelial marker occludin. These results are in agreement with some reports describing that cell motility and proliferation have been associated with activation of MEK/ERK by Insulin/IGF-I ligands [34]. In addition, our observations are in accordance with reports showing that the autocrine production of insulin-like growth factor-I (IGF-I) reduces occludin levels and alters paracellular transport in mammary epithelial cells in vitro [35].

Although we cannot exclude that IR/IGF-IR signaling pathways may affect other important factors, the combination of previous reports from our and other groups [17,19,20] with the present results support a close interplay between E-cadherin, its glycosylation with bisecting GlcNAc N-glycans and IR/IGF-IR signaling in the process of tumour cell invasion. Corroborating this hypothesis, the stimulation of mock-transfected cells with insulin and IGF-I (Figures S2, S3 and S4) did not affect the fibronectin mRNA transcription levels nor the invasive phenotype (Figure S3). In addition, no changes were observed on the β-catenin cellular localization after stimulation of mock cells with insulin or IGF-I (Figure S4). Moreover, in MKN45 gastric cancer cells transfected with MGAT5 (a cellular model known to induce changes in E-cadherin glycosylation and consequently in tumour cell behaviour[17]), we have demonstrated that the overexpression of GnT-V leads to an increased expression of the Insulin Receptor (Figure S3 E), which further supports the relationship between post-translational modifications of E-cadherin and IR/IGF-IR signaling in the process of tumour cell invasion. Our observations are the first to clearly demonstrate that bisecting GlcNAc *N*-glycans expression appears to be regulated by the interplay of E-cadherin and IR/IGF-IR signaling, which are known to be crucial in the process of epithelial-mesenchymal transitions and consequetly in the process of tumor cell invasion and metastases. We here propose that E-cadherin induces the expression of bisecting GlcNAc N-glycans in the absence (switch-off) of IR/IGF-IR signaling, which in turn will contribute to the stabilization of E-cadherin at the cell membrane, ensuring an epithelial-like phenotype, ultimatly promoting tumor suppression [17,36]. The signaling activation of IR/IGF-IR pathway, through the activation of Ras/Raf/MEK/ERK, induces a significant decreased expression of bisecting GlcNAc *N*-glycans in general and specifically on E-cadherin, which in turn leads to a destabilization of E-cadherin at the cell mebrane together with increased expression of mesenchymal markers. This alteration of E-cadherin glycosylation through IR/IGF-IR signaling activation favors an increased tumor cell invasion capability (Figure 8, proposed model).

In conclusion, our findings strongly support that Insulin/IGF-I signaling is an appealing target with implication in the modulation of glycosylation of key molecules involved in tumor invasion, having therefore promising therapeutic aplications in epithelial cancers.

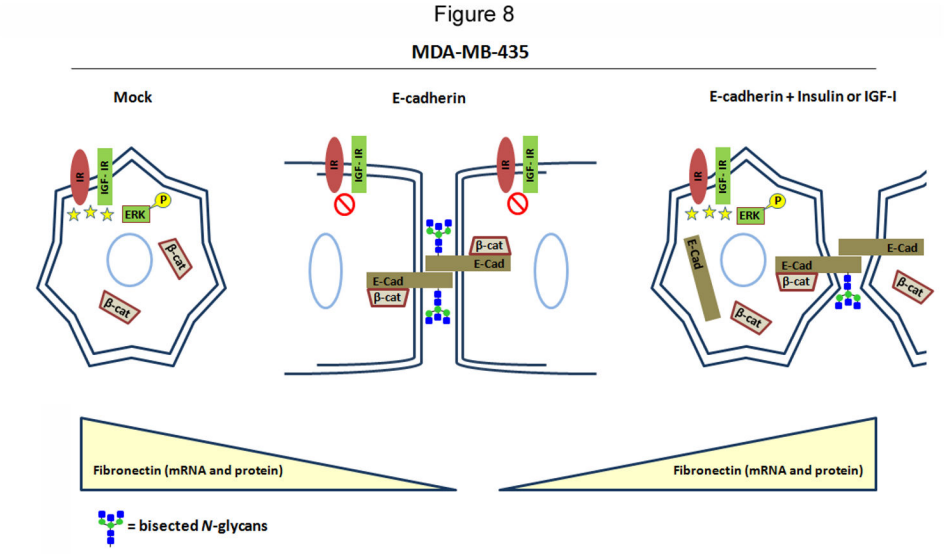

Figure 8. Proposing model for the interplay between E-cadherin, IR/IGF-IR, and bisecting GlcNAc *N*-glycans on the stabilization of both cell-cell adhesion and epithelial-like phenotype. The figure summarizes our findings and shows that exogenous E-cadherin expression leads to an inhibition of IR/IGF-IR signaling, concomitantly with increased levels of bisecting GlcNAc *N*-glycans expression which were previously shown to stabilizes adherens-junctions, ensuring an epithelial-like phenotype [18,35]. Stimulation with insulin or IGF-I activates IR/IGF-IR signaling and downstream protein ERK 1/2, promoting a decreased expression of bisecting GlcNAc structures in general and specifically on E-cadherin which was previously shown to destabilize cell-cell adhesion [18], leading to an invasive phenotype. Concomitantly, it was observed an increased expression of the mesenchymal marker fibronectin and cytoplasmic β-catenin and E-cadherin.

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Supporting Information

Figure S1. Effects of exogenous E-cadherin expression on the phosphoproteome profile. Total cell lysates from MDA-MB-435+mock and MDA-MB-435+E-cad were obtained and analyzed by Phospho-RTK array using 300 µg of proteins. The phosphor-RTK coordinates are shown on the top of figure illustrating the localization of the spots containing immobilized antibodies on the nitrocellulose membrane. The bar graphs show the relative densities of black dots. The most pronounced changes are observed in IR (coordinates B17 and B18) and IGF-IR (coordinates B19 and B20).
(TIF)

Figure S2. Effects of stimulation of Mock-transfected cells with insulin and IGF-I on the phosphorylation of tyrosine kinase receptors and downstream proteins. (A,B) Total cell lysates from MDA-MB-435+mock cells and MDA-MB-435+mock stimulated (24h) with insulin or IGF-1 were obtained and analyzed by Western-blot for phospho-IR(Tyr1150-51)/phospho-IGF-IR(Tyr1135-36), IR, IGFR, Akt, phospho-Akt (Ser 473), ERK 1/2, phospho-ERK 1/2, β-catenin and E-cadherin. Increased phosphorylation levels of IR, IGF-IR, ERK 1/2 and Akt were observed after stimulation with insulin or IGF-I. Tubulin was used as a loading control.
(TIF)

Figure S3. Effects of stimulation of Mock-transfected cells with insulin and IGF-I on cell invasion. (A) Representative images of cell invasion through Matrigel using 8 mm pore of a polycarbonate membrane. Nuclei were stained with DAPI. No significant differences were observed on cellular invasion upon insulin and IGF-I stimulation of mock-transfected cells. (B) The bar graph shows the amount of cells/field. **Effects of stimulation with insulin and IGF-I on the fibronectin protein and mRNA expression levels, respectively**. (C) and (D) A slight increase of fibronectin protein expression levels were observed after stimulation with insulin or IGF-I, however, no significant changes were observed at the mRNA transcription levels after stimulation of MDA-MB-435+E-mock cells with insulin and IGF-I . **Effects of overexpression of MGAT5 on the IR expression levels of MKN45 cell line**. (E) Total cell lysates from MKN45+mock and MKN45+MGAT5 were obtained and analyzed by Western blot for IR. An increased expression of IR were observed after overexpression of MGAT5. Tubulin was used as a loading control.
(TIF)

Figure S4. Subcellular localization of E-cadherin and β-catenin of Mock-transfected cells stimulated with insulin and IGF-I. Cell monolayers from MDA-MB-435+mock stimulated (24h) with insulin or IGF-1 were fixed and stained for E-cadherin, β-catenin and nucleus (DAPI). No significant differences were observed on the β-catenin subcellular localization after insulin or IGF-I stimulation. The representative images were obtained by fluorescence microscopy. Bar = 10 µm.
(TIF)

Acknowledgements

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Author Contributions

Conceived and designed the experiments: JCMdFJ CAR SSP. Performed the experiments: JCMdFJ SC JC AMD. Analyzed the data: JCMdFJ CAR SSP CO JAMD. Contributed reagents/materials/analysis tools: PO RS. Wrote the manuscript: JCMdFJ SSP.

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Chapter III

Preventing E-cadherin aberrant N-glycosylation at Asn-554 improves its critical function in gastric cancer

Oncogene (2015), 1–13

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ORIGINAL ARTICLE

Preventing E-cadherin aberrant *N*-glycosylation at Asn-554 improves its critical function in gastric cancer

S Carvalho[1,2], TA Catarino[1], AM Dias[1,2], M Kato[3], A Almeida[4,5], B Hessling[6], J Figueiredo[1], F Gärtner[1,2], JM Sanches[7], T Ruppert[6], E Miyoshi[8], M Pierce[9], F Carneiro[1,10,11], D Kolarich[4], R Seruca[1,10], Y Yamaguchi[3], N Taniguchi[3], CA Reis[1,2,10] and SS Pinho[1,2]

E-cadherin is a central molecule in the process of gastric carcinogenesis and its posttranslational modifications by *N*-glycosylation have been described to induce a deleterious effect on cell adhesion associated with tumor cell invasion. However, the role that site-specific glycosylation of E-cadherin has in its defective function in gastric cancer cells needs to be determined. Using transgenic mice models and human clinical samples, we demonstrated that *N*-acetylglucosaminyltransferase V (GnT-V)-mediated glycosylation causes an abnormal pattern of E-cadherin expression in the gastric mucosa. *In vitro* models further indicated that, among the four potential *N*-glycosylation sites of E-cadherin, Asn-554 is the key site that is selectively modified with β1,6 GlcNAc-branched *N*-glycans catalyzed by GnT-V. This aberrant glycan modification on this specific asparagine site of E-cadherin was demonstrated to affect its critical functions in gastric cancer cells by affecting E-cadherin cellular localization, *cis*-dimer formation, molecular assembly and stability of the adherens junctions and cell–cell aggregation, which was further observed in human gastric carcinomas. Interestingly, manipulating this site-specific glycosylation, by preventing Asn-554 from receiving the deleterious branched structures, either by a mutation or by silencing GnT-V, resulted in a protective effect on E-cadherin, precluding its functional dysregulation and contributing to tumor suppression.

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INTRODUCTION

Epithelial cadherin (E-cadherin) is a calcium-dependent cell–cell adhesion molecule with a critical role in epithelial tissue morphogenesis.[1–7] The mature E-cadherin protein is organized into three major structural domains: an ectodomain of about 550 amino acids comprised of five tandemly repeated subdomains (EC1–EC5), a single transmembrane domain and a short cytoplasmic domain of about 150 amino acids. The human E-cadherin ectodomain is comprised of four potential *N*-glycosylation sites: two putative sites located in the EC4 subdomain (Asn-554 and Asn-566) and the remaining two potential sites in the EC5 subdomain (Asn-618 and Asn-633).[6]

Epithelial cell–cell adhesion is achieved through homophilic interactions between cadherin molecules of neighboring cells. The extracellular domains of E-cadherins on the same cell surface establish lateral or *cis*- interactions and then among adjacent cells to form *trans* adhesive bonds, leading to the formation of a zipper-like structure.[8–10] Cell–cell adhesion is further accomplished through the molecular interaction of the cytoplasmic domain of E-cadherin and the catenins β-, γ-, α- and p120-catenin. The stability of this cadherin–catenin complex, and its interaction with the actin cytoskeleton, forms the core adherens junctions, which is essential for cell–cell adhesion and precludes individual epithelial cell motility, thus providing a normal and homeostatic tissue architecture.[11,12]

The functional inactivation or downregulation of E-cadherin is considered to be a hallmark of the epithelial carcinogenic process, being closely associated with tumor cell invasion and metastases.[13] Several mechanisms have been proposed to explain the loss of function of E-cadherin in cancer,[7] and among those mechanisms, the modification of E-cadherin by glycosylation has been demonstrated to be instrumental for the regulation of E-cadherin functions in the context of cancer.[14,15] Moreover, O-mannosylation of E-cadherin was recently demonstrated to be important for E-cadherin-mediated cell–cell adhesion.[16,17]

In fact, during malignant transformation, the glycosylation profile of E-cadherin undergoes a significant alteration,[18] with implications for its biological functions. Of those glycosylation alterations, two are generally considered to be fundamental for the regulation of the protein: the bisecting GlcNAc *N*-glycan structures (catalyzed by the *N*-acetylglucosaminyltransferase III (GnT-III) glycosyltransferase), and the β1,6 GlcNAc-branched *N*-glycan structure (catalyzed by the GnT-V glycosyltransferase). These two key glycan structures were recently found to precisely control, in an opposite manner, the functions of E-cadherin in cancer cells.[15,19–24] E-cadherin has different *N*-glycosylation sites that are potentially responsible for controlling its biological

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functions. Under normal physiological circumstances, E-cadherin was described to be primarily modified with high-mannose/hybrid *N*-glycans;[25,26] however, the key site(s) that are crucial for the regulation of its functions in the context of cancer remain to be determined.

In this study, we report that E-cadherin becomes functionally impaired when GnT-V glycosyltransferase catalyzed modification of site 1 (Asn-554) with β1,6 GlcNAc-branched *N*-glycan structures. Further, the presence of a mutation of this specific *N*-glycosylation site (which abrogates the availability of this site to be modified by GnT-V) has a protective effect on E-cadherin function, preventing its dysregulation.

RESULTS

Impact of GnT-V-mediated glycosylation in E-cadherin expression in the mice stomach and in human gastric carcinoma

In the present study, we used GnT-V transgenic mouse models to show that differential expression of E-cadherin in the gastric mucosa is influenced by the presence of GnT-V. We used two transgenic mouse models: *MGAT5* knockout (with no GnT-V activity) and *MGAT5* transgenic mice that overexpress GnT-V activity. We observed that the gastric mucosa of the wild-type (WT) mice showed a moderate expression of β1,6 GlcNAc-branched structures detected by the *biotinylated Phaseolus vulgaris leucoagglutinin* (L-PHA) lectin. The gastric mucosa of the *MGAT5* knockout mice showed no L-PHA reactivity (Figure 1a). On the other hand, mice overexpressing GnT-V showed high levels of expression of β1,6 GlcNAc-branched structures in the gastric mucosa. Regarding E-cadherin localization, *MGAT5* knockout mice showed E-cadherin normally expressed in the basolateral cell membrane of gastric epithelial cells. In GnT-V-overexpressing mice, E-cadherin was also displayed in the cytoplasm of cells at the neck zone and in deep glands of the gastric mucosa (Figure 1a). No major histopathological lesions were observed in the gastric mucosa of these mouse models.

Similarly, the levels and profile of E-cadherin modifications with the aberrant β1,6-branched *N*-glycans (catalyzed by GnT-V) in human gastric carcinomas (diffuse gastric cancer sub-type) and the correlation with prognostic features were demonstrated by performing *in situ* Proximity Ligation Assay (PLA) technique (Figure 1b). PLA is a technology that extends the capabilities of traditional immunoassays to include direct detection of proteins, protein interactions and posttranslational modifications such as glycosylation with high specificity and sensitivity.[27] Our results showed that gastric carcinoma cells display a marked increase of positive PLA signals demonstrating an increased modification of E-cadherin with β1,6-branching *N*-glycans. The normal gastric mucosa was either negative or showed a weak PLA signal, suggesting that E-cadherin is not modified by GnT-V-mediated branching glycans in normal gastric cells (Figure 1b). Furthermore, and in order to evaluate whether the GnT-V-mediated glycosylation of E-cadherin is correlated with prognosis, a series of 19 patients with diffuse gastric cancer was analyzed and associated with prognostic variables. Patients positive for E-cadherin+β1, 6-branched *N*-glycans (positive PLA) display a poorer survival rate (assessed by Kaplan–Meier survival analysis) when compared with diffuse gastric cancer patients negative for PLA E-cadherin/L-PHA ($P = 0.093$; Figure 1, Graphic b1). Additionally, we further showed that all patients who do not survive were positive for PLA E-cadherin/ L-PHA, as represented in Table 1 ($P = 0.077$).

These results demonstrate the importance of this specific E-cadherin aberrant glycoform *in vivo* in the pathogenesis of gastric carcinoma.

Bioinformatics evaluation of E-cadherin N-glycosylation site occupancy

Glycosylation is known to be a cell- and tissue-specific event and the levels and pattern of glycosylation of a specific protein vary accordingly with cell and tissue location[28] (Supplementary Figure S1). *N*-linked glycosylation does not occur at every potential glycosylation site. Depending on the protein and on the physiopathological context, some glycosylation sites are more important than others for the regulation of protein function.[29]

Using an *in silico* bioinformatics analysis that takes three main criteria into consideration, E-cadherin *N*-glycosylation site occupancy could be predicted. The first criterion is based on the species homology of the E-cadherin peptide sequence (Figure 2a). A comparison of the E-cadherin peptide sequence between different species revealed that the *N*-glycosylation sites Asn-554 (site 1) and Asn-633 (site 4) are the most conserved among species.

The second criterion used in the evaluation is the site-specific modification of *N*-linked glycans through the solvent accessible surface area (ASA) calculation of four Asn residues. Very low ASA values at Asn residue indicate a reduced probability of *N*-glycans attachment. As shown in Figure 2b, the potential *N*-glycosylation site Asn-618 (site 3) displays a low ASA value, suggesting a low probability of being *N*-glycosylated.

The third criterion is the evaluation of the β-turn propensity at the Asn-*x*-Ser/Thr acceptor sequence (Figure 2c). The variable *x* represents the sequence position around the sequon and a high value of β-turn propensity at an Asn residue can positively affect the spatial configuration of Asn and Ser/Thr side chains towards the approach of an oligosaccharyltransferase. For $x = 0$ (Asn residue), Asn-554 (site 1) and Asn-633 (site 4) show higher β-turn propensity values demonstrating a high probability for the occurrence of *N*-glycosylation. It has also been reported that the presence of Pro at $x = 3$ position inhibits *N*-glycosylation.[30,31] Taken these issues into account, Asn-566 (site 2) and Asn-618 (site 3) are not likely to be glycosylated.

Overall, the combination of these *in silico* parameters (Figure 2d) suggests that *N*-glycosylation is likely to occur at Asn-554 (site 1) and Asn-633 (site 4) and not likely to occur in the other two putative sites (sites 2 and 3).

E-cadherin Asn-554 (site 1) and Asn-633 (site 4) are occupied with complex- and high-mannose/hybrid-type N-glycans, respectively

In order to further characterize *in vitro* the E-cadherin *N*-glycosylation site occupancy, we applied site-directed mutagenesis to generate different E-cadherin *N*-glycan mutants lacking selected potential *N*-glycosylation sites by replacing asparagine for glutamine residues in each *N*-glycosylation consensus sequence (NXS/T) either individually or in different combinations (Figure 3a).

The different E-cadherin *N*-glycan mutants were transfected into gastric carcinoma AGS cell line (that lacks endogenous E-cadherin both at mRNA and protein levels[32]), and the mobility shift of the E-cadherin band was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; Figure 3b). Concerning the individual E-cadherin *N*-glycan mutants (M1, M2, M3 and M4), the results showed that the E-cadherin mutant M1 (lacking site 1, Asn-554) revealed the most pronounced mobility shift followed by the E-cadherin mutant M4 that displayed a slight mobility compared with E-cadherin WT. E-cadherin mutants M2 and M3 did not exhibit a mobility shift compared with the WT. These results suggest that sites 1 (Asn-554) and 4 (Asn-633) are *N*-glycosylated. In addition, and consistent with the bioinformatics prediction, site 2 (Asn-566) and site 3 (Asn-618) are not occupied with *N*-glycans. Validating these observations, the multiple E-cadherin *N*-glycan mutants M1234 (total *N*-glycan mutant) and M14 (with Asn-554 and Asn-633 not present) showed the same

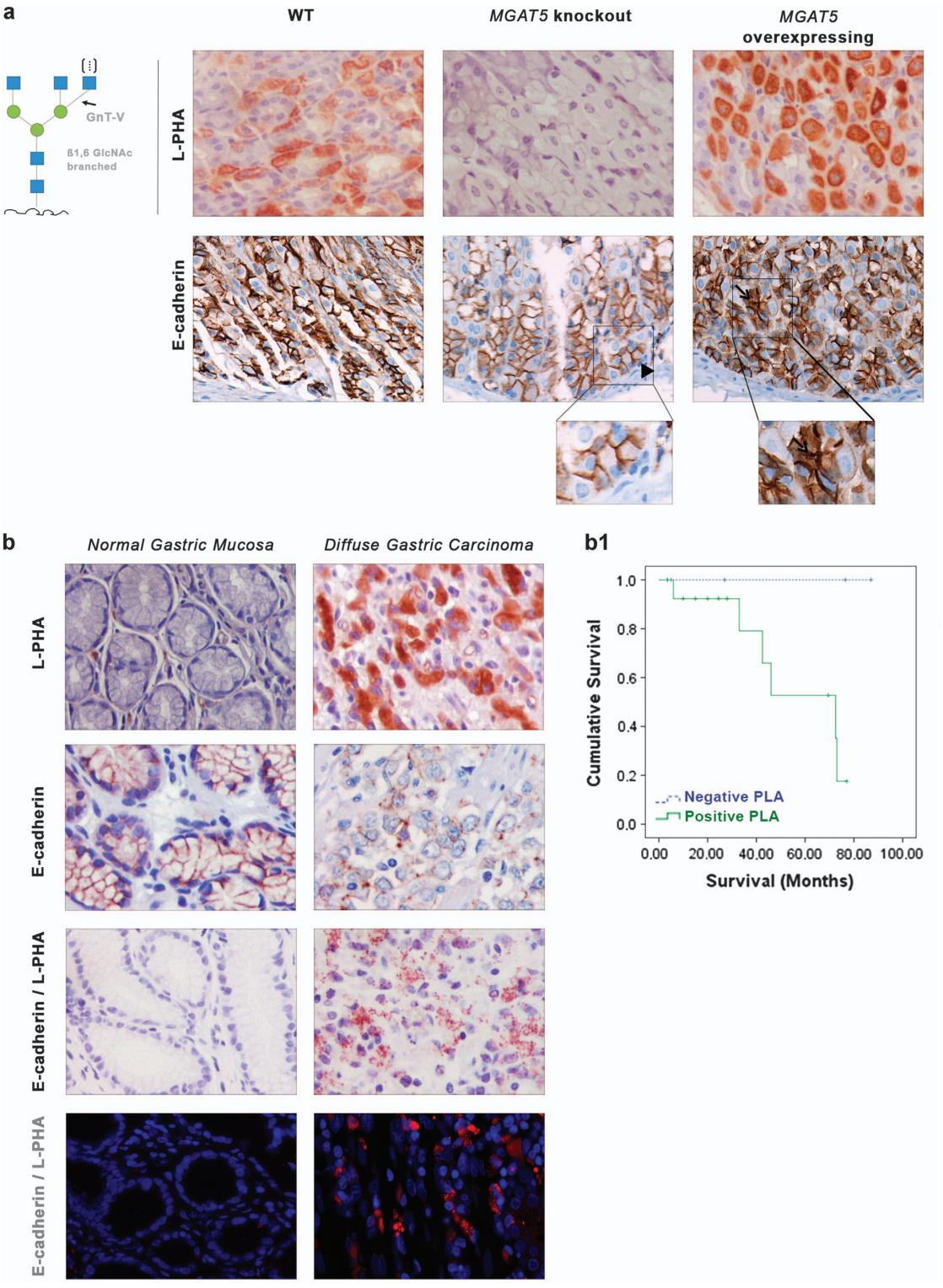


Figure 1. Evaluation of the expression of E-cadherin and β1,6 GlcNAc-branched structures in the gastric mucosa of *MGAT5* knockout and *MGAT5* overexpressing and in human normal gastric mucosa versus gastric carcinoma. (**a**) L-PHA histochemistry detecting the β1,6 GlcNAc-branched *N*-glycans catalyzed by GnT-V showed a moderate expression of the β1,6-branched structures in the gastric mucosa of WT mice. No positive L-PHA reactivity was observed in the gastric mucosa of *MGAT5* KO mice, whereas a clear overexpression of β1,6 GlcNAc-branched structures was detected in *MGAT5* transgenic gastric mucosa. *MGAT5* KO mice showed a normal E-cadherin expression at the basolateral cell surface (arrowhead). In *MGAT5* transgenic mice, immunoexpression of E-cadherin was displayed aberrantly in the cytoplasm of mucous neck cells (not shown) and in deep glands of the gastric mucosa (arrow). (**b**) *In situ* PLA showed weak/absence PLA signal in normal gastric mucosa and a marked positivity of PLA signal (brown dots for brightfield and red dots for immunofluorescence) in neoplastic cells from gastric carcinoma, demonstrating a profound modification of E-cadherin with the β1,6 GlcNAc-branched structures in human gastric cancer (×40, original magnification). (**b**1) Survival rates of patients with diffuse gastric cancer (DGC) accordingly with PLA signal (positive versus negative). Kaplan–Meier curves demonstrate the probability of overall survival for patients with DGC accordingly with the presence/absence of GnT-V-mediated aberrant glycosylation of E-cadherin ($P = 0.093$).

Table 1. Relationship between aberrant glycosylation of E-cadherin mediated by GnT-V and prognostic variables of diffuse gastric cancer patients

Variable	*PLA E-cadherin/L-PHA*		
	Negative	*Positive*	P
	No. of patients (%)		
Depth of invasion			
pT1	2 (25%)	6 (75%)	0.298
pT2	0 (0%)	4 (100%)	
pT3–4	3 (42.9%)	4 (57.1%)	
Lymph node involvement			
Absent (pN0)	2 (28.6%)	5 (71.4%)	0.865
Present (pN+)	3 (25%)	9 (75%)	
Lymphatic permeation			
Negative	2 (22.2%)	7 (77.8%)	0.701
Positive	3 (30%)	7 (70%)	
Venous invasion			
Negative	2 (18.2%)	9 (81.8%)	0.345
Positive	3 (37.5%)	5 (62.5%)	
Status			
Alive	5 (38.5 %)	8 (61.5%)	0.077
Dead	0 (0%)	6 (100%)	

Abbreviations: Gnt-V, *N*-acetylglucosaminyltransferase V; L-PHA, *biotinylated Phaseolus vulgaris leucoagglutinin*; PLA, Proximity Ligation Assay.

mobility shift that was slightly higher than that for E-cadherin M1. E-cadherin M23 did not display a mobility shift compared with E-cadherin WT, further suggesting that sites 2 and 3 are not *N*-glycosylated.

Next we determined the susceptibility of the E-cadherin *N*-glycan mutants towards different endoglycosidases, namely *PNGase F* and *Endo H*. As shown in Figure 3c, E-cadherin WT showed partial sensitivity to *Endo H* but, being predominantly *PNGase F*-sensitive, indicating that E-cadherin WT is modified with both complex and high-mannose/hybrid-type *N*-glycans. The E-cadherin mutant M234 showed a slight shift upon *Endo H* but exhibited a significant mobility shift after *PNGase F* digestion. On the other hand, treatment of M123 with *Endo H* resulted in a substantial mobility shift that was not further enhanced by *PNGase* F. These observations indicate that Asn-554 is modified mostly by complex-type structures, whereas Asn-633 appears to contain mainly high-mannose/hybrid-type *N*-glycans. This is also consistent with the higher mobility shift of M1 compared with M4 (Figure 3b).

These observations were further supported by a glycomics analysis of the released *N*-glycans of E-cadherin WT, M123 and M234 by porous graphitized carbon (PGC) nano-liquid chromatography–electronspray ionization tandem mass spectrometry (nanoLC-ESI MS/MS). The respective E-cadherin proteins were immunoprecipitated, SDS–PAGE separated and electro-blotted onto polyvinylidene difluoride (PVDF) membranes prior on-blot *PNGase F* digestion as described earlier.[33,34] E-cadherin WT and M234 showed higher levels of sialylated and complex type *N*-glycans, respectively (Figure 3d). E-cadherin WT showed the highest levels of complex type, triantennary *N*-glycans (Supplementary Figure S2, Supplementary Tables S1 and S2). These structures were found reduced in M123 and could not be detected in M234 (Supplementary Figure S2), further indicating the presence of tetra-antennary *N*-glycans in M234 (though these analyses do not allow an absolute glycan quantitation).

Regarding M134, M124 and M14 (all with a mutation at sites 1 and 4), no detectable changes were observed after *Endo H/PNGase F* treatment, supporting the hypothesis that Asn-566 (site 2) and Asn-618 (site 3) are not *N*-glycosylated. As expected, E-cadherin with all potential *N*-glycosylation sites mutated (M1234) was neither *Endo H* nor *PNGase F* sensitive. Taken together (see also Supplementary Figure S3), these results indicate that, in gastric cancer cells, E-cadherin is modified with mostly complex-type *N*-glycans at site 1 (Asn-554) and with mainly high-mannose/hybrid-type at site 4 (Asn-633), whereas sites 2 and 3 (Asn-566 and Asn-618) were not *N*-glycosylated. A statistical analysis suggests that glycosylation sites carrying highly processed complex-type glycans are significantly more solvent-accessible than those carrying less processed high-mannose-type glycans.[35] Consistent with this, ASA of Asn-633 is lower than that of Asn-554 (Figure 2b).

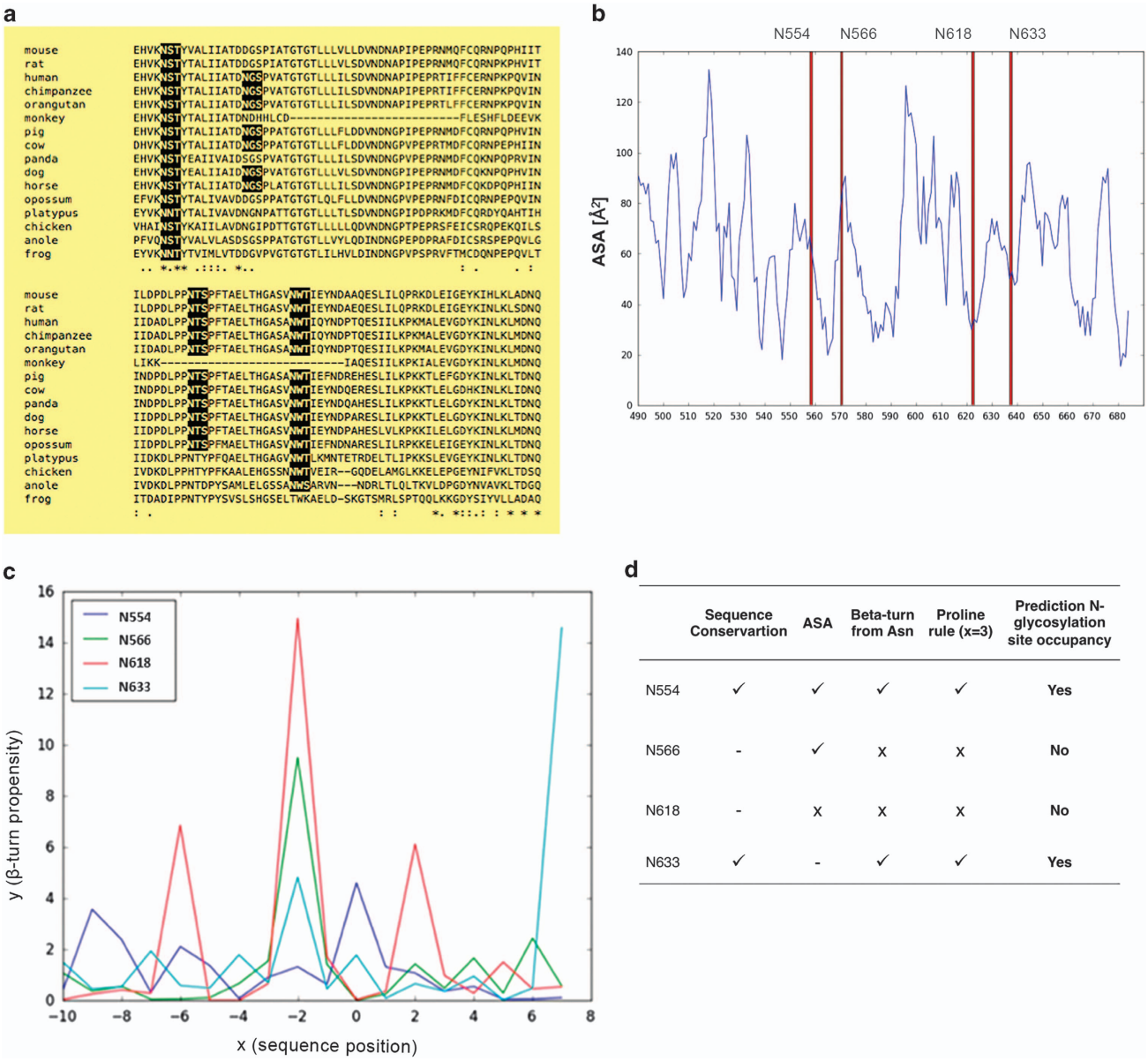
Site-specific occupancy of Asn-554 with complex type N-glycans has a deleterious effect on E-cadherin localization and dimerization

The glycosylation of E-cadherin with complex-type *N*-glycans, such as bisecting GlcNAc and the β1,6 GlcNAc-branched *N*-glycans structures, has been reported to precisely regulate E-cadherin functions in an opposing manner.[15,20] Having confirmed that site 1 (Asn-554) of E-cadherin is the one that is mainly occupied with complex-type *N*-glycans (Figure 3, Supplementary Figure S2), we proceeded to further evaluate the functional impact of this site-specific *N*-glycan occupancy by comparing the mutants M1 and M234, representing the absence versus the presence of a complex *N*-glycan on site 1, respectively. By immunofluorescence analysis, we evaluated the cellular localization of E-cadherin and cell morphology, and the results showed that AGS cells expressing the E-cadherin M1 mutant (Figure 4a) or M123 (Supplementary Figure S4A) exhibited a membrane localization of E-cadherin that showed a more focused membrane staining than the E-cadherin WT (as revealed by confocal microscopy). By contrast, AGS cells expressing E-cadherin M234 (or E-cadherin M4, Supplementary Figure S4A) displayed an aberrant E-cadherin expression with mislocalization into the cytoplasm. Quantitative analyses of *in situ* immunofluorescence images that consider internuclear profiles further showed a focused membrane localization of E-cadherin after mutation at Asn-554 (E-cadherin M1) and a decreased E-cadherin membrane expression in M234 (Figure 4A1). Regarding the E-cadherin M2, M3 and M23 mutants, no alterations were observed in the cellular localization of E-cadherin compared with E-cadherin WT (Supplementary Figure S4A).

We then attempted to determine whether *cis*-dimer formation of E-cadherin was affected by the occupancy/mutation of site 1 with complex-type *N*-glycans, by performing a *cis*-dimerization assay using BS^3, a membrane-impermeable chemical cross-linker. The formation of E-cadherin *cis*-dimers was observed after AGS cells expressing E-cadherin WT, M1 or M234 were treated with BS^3 (Figure 4b). However, the levels of E-cadherin *cis*-dimerization were significantly higher in E-cadherin M1 than in E-cadherin WT and M234 (Figure 4B1) or than in M4 (Supplementary Figures S4B and B1), indicating that the mutation of Asn-554 (M1) enhanced the formation of E-cadherin *cis*-dimers.

In addition, a calcium switch assay was performed (Supplementary Figure S5). These results showed that the calcium-binding property appear to be independent of E-cadherin glycosylation; however, it is observed that E-cadherin M234 showed a more evident impact in the calcium-binding property of E-cadherin.

Consistent with this, the deletion of site 1 (M1) induced a significantly increased rate of cell–cell adhesion together with the formation of larger compact cellular aggregates when compared with E-cadherin WT and M234 (Figures 4c and c1; Supplementary Figure S4C).



	Sequence Conservartion	ASA	Beta-turn from Asn	Proline rule (x=3)	Prediction N-glycosylation site occupancy
N554	✓	✓	✓	✓	Yes
N566	-	✓	x	x	No
N618	-	x	x	x	No
N633	✓	-	✓	✓	Yes

Figure 2. Prediction of E-cadherin *N*-glycan site occupancy based on bioinformatics. (**a**) Species homology of the E-cadherin peptide sequence. The potential *N*-glycosylation sites Asn-554 and Asn-633 are well conserved. (**b**) Calculation of ASA of the four Asn residues. The potential *N*-glycosylation site Asn-618 displays a low ASA value. (**c**) Evaluation of β-turn propensity of the region adjacent to the Asn-X-Ser/Thr acceptor sequence. Horizontal axis (*x*) indicates sequence position around the sequon ($x = 0$: Asn), and vertical axis (*y*) shows the β-turn propensity. For $x = 0$, high values of β-turn propensity mean high possibility for *N*-glycosylation to occur. (**d**) Prediction of E-cadherin *N*-glycan's occupancy. Asn-554 and Asn-633 are the two putative *N*-glycosylation sites more likely to be occupied.

Site-specific occupancy of Asn-554 with complex-type N-glycans impairs a competent adhesion complex formation

We further evaluated whether the mutation of Asn-554 and/or the occupancy of this specific *N*-glycan site could affect the molecular organization and assembly of the cadherin–catenin complex. No significant differences were observed in the total expression levels of the catenins, β- and p120-catenin, among the different E-cadherin *N*-glycan mutants (Figure 4d). However, an increased E-cadherin/β-catenin interaction was verified in E-cadherin M1 in comparison with E-cadherin WT (0.7-fold) and a significantly decreased interaction was found between E-cadherin and β-catenin when Asn-554 (site 1) was *N*-glycosylated (M234 or M4, Figures 4e and e1; Supplementary Figures 4D and D1). In addition, we verified a 1.3- and 0.9-fold increased interaction between E-cadherin and p-120-catenin when Asn-554 (site 1) was mutated (M1) when compared with E-cadherin WT and E-cadherin M234, respectively, suggesting that mutation of site 1 resulted in an increased recruitment of catenins by E-cadherin (Figures 4e and e1). These differences in the recruitment of catenins by E-cadherin reflect the positive impact on the assembly and stability of the cadherin–catenin complex induced by the mutagenesis of Asn-554 (site 1).

Preventing Asn-554 site-specific glycosylation improves E-cadherin functions

Having demonstrated the deleterious effects of site 1 *N*-glycan occupancy for the biological functions of E-cadherin in gastric tumor cells (Figure 4), we further identified the specific type of complex *N*-glycans that are attached to Asn-554. Figure 5a

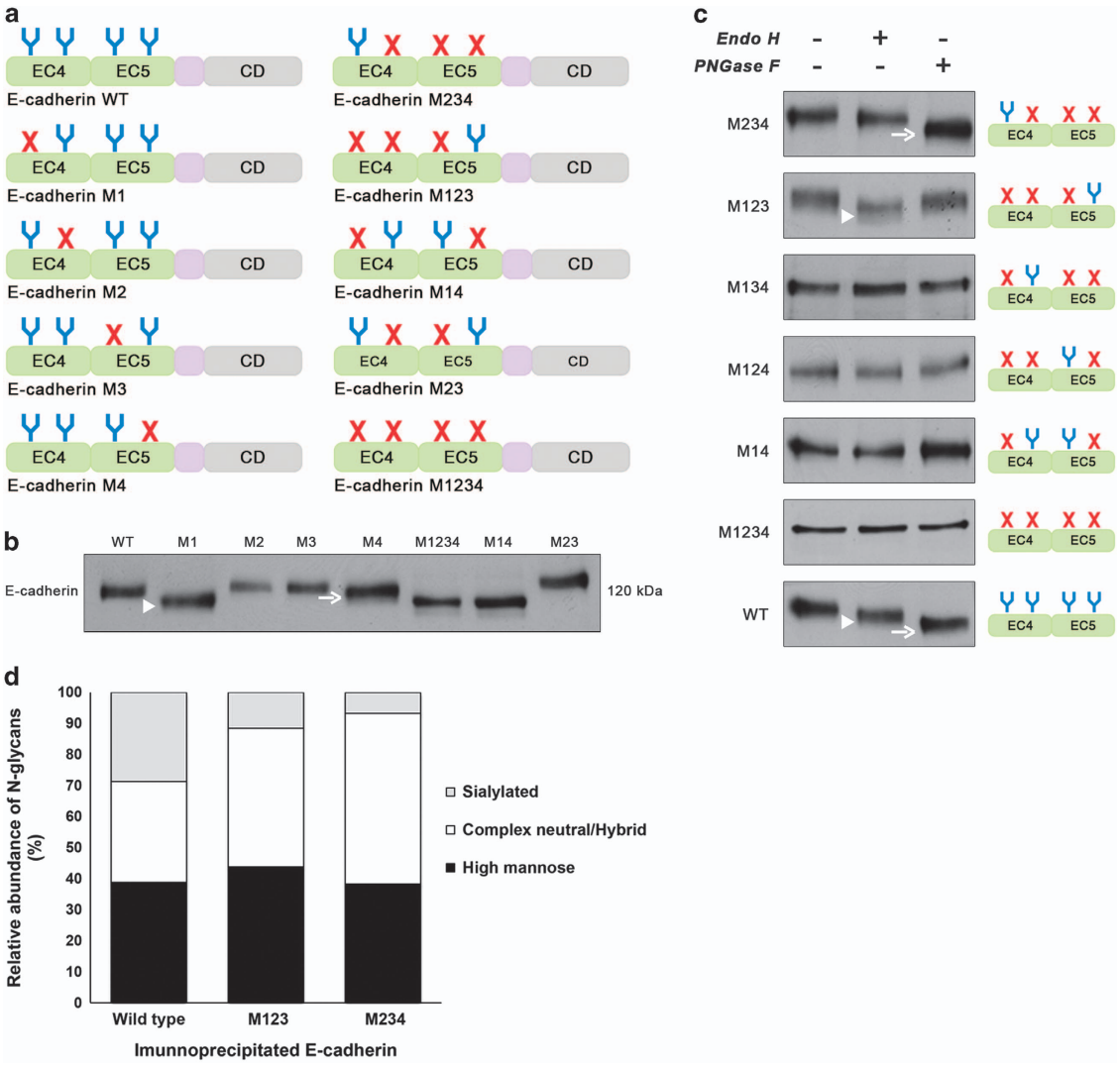


Figure 3. *N*-glycosylation sites Asn-554 and Asn-633 are *N*-glycosylated being occupied with complex-type *N*-glycans and high-mannose/hybrid- type *N*-glycans, respectively. (**a**) Schematic representation of different E-cadherin *N*-glycan mutants lacking selected putative *N*-glycosylation sites by replacing asparagine residue with glutamine in each *N*-glycosylation consensus sequence (NXS/T) either individually or in different combinations. EC4, subdomain EC4; EC5, subdomain EC5; CD, cytoplasmic domain; E-cadherin WT, E-cadherin wild type; E-cadherin M1, lacking Asn-554; M2, lacking Asn-566; M3, lacking Asn-618; M4, lacking Asn-633; M14, lacking Asn-554 and Asn-633; M23, lacking Asn-566 and Asn-618; M234, lacking Asn-566, Asn-618, and Asn-633; M123, lacking Asn-554, Asn-566, Asn-618; M1234, lacking Asn-554, Asn-566, Asn-618, and Asn-633. (**b**) Evaluation of the mobility shift of E-cadherin *N*-glycan mutants in AGS cells compared with WT. E-cadherin *N*-glycan mutant M1 and M4 showed an increased mobility compared with E-cadherin WT. No detectable changes were observed regarding cadherin M2 and M3. E-cadherin M1 (arrowhead) displayed a higher mobility shift than E-cadherin M4 (arrow). (**c**) Characterization of *N*-glycosylation profile of E-cadherin *N*-glycan mutants in AGS cells. Total cell lysates from selected transfectants were treated with Endo H and PNGase F and analyzed for mobility shifts by western blotting. E-cadherin WT was sensitive to Endo H (arrowhead) and PNGase F (arrow) being modified with high-mannose-, hybrid- and complex-type *N*-glycans. E-cadherin M234 was mostly sensitive to PNGase F (arrow) and resistant to Endo H while E-cadherin M123 was predominantly Endo H-sensitive (arrowhead). See also Supplementary Figure S3. (**d**) N-glycomics analysis using PGC nanoLC ESI MS/MS analyses showed E-cadherin WT to carry high-mannose, complex neutral and complex sialylated *N*-glycans in similar amounts. E-cadherin WT and M234 showed higher levels of sialylated and complex-type *N*-glycans, respectively. The data presented is referred to one biological replicate, which according to the well-established methodology, can be considered reproducible and significant. See also Supplementary Figure S2 and Supplementary Tables S1 and S2.

demonstrates that E-cadherin M234 showed an increased reactivity with the L-PHA lectin compared with M1, which is indicative of the presence of β1,6 GlcNAc-branched structures at Asn-554 (site 1). Both E-cadherin WT and M234 show similar β1,6 GlcNAc-branched expression patterns, as both retain the Asn-554 complex glycan. In contrast, no positive reactivity with L-PHA on the E-cadherin band in the M1 mutant was observed, showing that site 1 is specifically modified with β1,6 GlcNAc-branched structures. Despite that it was not possible to unambiguously substantiate the β1,6 GlcNAc nature of the branching in M234 with the minimal amounts of E-cadherin material available for glycomic analyses, the triantennary *N*-glycans were only detected in E-cadherin WT and M123 and not in M234. Nevertheless, the combined data presented here clearly indicate that also a subtle increase of a specific structure feature on E-cadherin induces a strong functional response.

To confirm these results, we performed silencing of the *MGAT5* gene using the small interfering RNA (siRNA) technique (Figure 5b). As shown in Figure 5c, the knockdown of GnT-V resulted in a significant decrease in the expression of β1,6 GlcNAc-

branched structures (decreased L-PHA reactivity) in total cell lysates compared with non-silenced (NS) cells. The decreased L-PHA reactivity was also observed in total cell lysates of AGS Mock siRNA cells when compared with NS cells (Supplementary Figure S6). As a consequence of the GnT-V knockdown, we observed a competitive increase in the expression of bisecting GlcNAc structures (increased *biotinylated Phaseolus vulgaris erythroagglutinin* (E-PHA) reactivity) in the total cell lysates of the GnT-V knockdown cells compared with NS cells, as previously described. The silencing of *MGAT5* led to a decrease in the reactivity of E-cadherin M234 with the L-PHA lectin compared with the control (NS), which further supports the conclusion that site 1 is specifically modified with β1,6 GlcNAc-branched structures catalyzed by GnT-V enzymatic activity (Figure 5d). Furthermore, our results showed that, upon *MGAT5* knockdown, the gastric tumor cells expressing E-cadherin M234+siGnT-V displayed a notable change of its pattern of expression, showing a decreased cytoplasmic staining and an increase in membrane expression compared with NS cells (Figure 5e). In addition, we also demonstrated that, after the knockdown of GnT-V, the ratio of E-cadherin *cis*-dimers in gastric tumor cells expressing E-cadherin M234+siGnT-V increased to levels similar to that for E-cadherin M1 (Figures 5f and f1), suggesting that the inhibition of GnT-V-mediated modification with β1,6 GlcNAc-branched structures at Asn-554 (site 1) induces increased E-cadherin *cis*-dimer formation and, consequently, the strengthening of cell–cell adhesion.

These results reveal that the GnT-V selectively modifies *N*-glycosylation site 1 (Asn-554) on E-cadherin in the context of gastric tumor cells and that its silencing clearly increases E-cadherin-mediated cell–cell adhesion and expression.

DISCUSSION

In the present study, using two different *MGAT5* mouse models, we showed that the pattern of E-cadherin expression in gastric mucosa cells depends on the presence of β1,6 GlcNAc-branched *N*-glycans that are formed as the result of enzymatic catalysis by GnT-V (*MGAT5*). *MGAT5* transgenic mice (which overexpress GnT-V-mediated glycosylation) display an abnormal pattern of E-cadherin cellular expression in gastric epithelial cells that overexpress β1,6 GlcNAc-branched *N*-glycans compared with *MGAT5* knockout mice (with absence GnT-V-mediated enzymatic activity). Additionally, we verified that human diffuse gastric carcinoma is characterized by an aberrant pattern of E-cadherin *N*-glycosylation mediated by GnT-V. Moreover, we observed that diffuse gastric cancer patients displaying an aberrant glycosylation of E-cadherin mediated by GnT-V tend to exhibit a poorer survival rate when compared with gastric cancer patients without GnT-V-mediated glycosylation on E-cadherin.

We further investigated the specific *N*-glycosylation site(s) that are required for controlling E-cadherin functions in gastric cancer, particularly the key E-cadherin *N*-glycosylation site that can be modified with the deleterious β1,6 GlcNAc-branched structures catalyzed by the action of the GnT-V glycosyltransferase. Through combination of bioinformatics prediction tools with mutagenesis and glycans enzymatic digestion approaches as well as glycomic analyses, our results consistently revealed that, among the four potential *N*-glycosylation sites available in the extracellular domain of E-cadherin, Asn-554 (site 1) and Asn-633 (site 4) are *N*-glycosylated, respectively, with complex-type and high-mannose *N*-glycans structures. Conversely, Asn-566 (site 2) and Asn-618 (site 3) of E-cadherin are not occupied with *N*-glycans, suggesting that these *N*-glycosylation sites are not critical for controlling the biological functions of E-cadherin in gastric tumor cells. Consistent with our observations, previous reports have shown that E-cadherin from a CHO cell line is modified with *N*-glycans at Asn-554 and Asn-633[ref. 36] and that Asn-633 is modified with high-mannose *N*-glycans that were demonstrated to be important for the folding of E-cadherin.[14] In addition, our glycomics data showed that E-cadherin WT carries triantennary, sialylated *N*-glycans, whereas the glycosylation was found to be altered on the M123 and M234 mutants. These data indicate that site-specific mutations of the *N*-glycosylation sites on E-cadherin appear to alter the protein conformation and thus also the accessibility for the glycosyltransferases required for the formation of described *N*-glycans.

We clearly demonstrated that site 1 (Asn-554) in the extracellular domain of E-cadherin is the selected site modified with the β1,6 GlcNAc-branched structures and the key site for the functional regulation of E-cadherin in gastric tumor cells. Whenever site 1 (Asn-554) is modified with β1,6 GlcNAc-branched structures, the localization and functions of E-cadherin are impaired (Figure 6) by: compromising its *cis*-dimerization capability, by decreasing the rate of cell–cell aggregation, and also by interfering in the molecular assembly and stability of the adhesion complex, that altogether promotes its cellular mislocalization and non-functional role. In fact, a mutation of this specific site 1 (E-cadherin M1 or M123) resulted in a protective effect, precluding the functional impairment of E-cadherin through the recovery of E-cadherin-mediated cell–cell adhesion, an increased stability of adherens junctions and the correct membrane localization of the molecule. Similarly, the silencing of the *MGAT5* gene that encodes GnT-V also resulted in a restoration of E-cadherin expression and functions. Although the GnT-V knockdown is likely to affect various cell surface receptors, in this study we focused on E-cadherin, showing that upon GnT-V knockdown, the E-cadherin *N*-glycan mutant M234 (in which site 1 is available) loses the β1,6 GlcNAc-branched structures and consequently has increased *cis*-dimerization capability, increases recruitment of catenins and, importantly, it reaches the cell membrane, behaving similar to the M1 mutant. These results on M234 (and M4) mutant also suggest that loss of high-mannose glycan in site 4 may also contribute to this process, as it was previously shown the importance of this site for E-cadherin folding.[14] Therefore, blocking Asn-554 (site 1), either by mutation or by GnT-V silencing, resulted in a protective effect on E-cadherin to not become aberrantly glycosylated thus avoiding its functional dysregulation (Figure 6). Taken together, these observations are in agreement with the results using transgenic mice regarding an E-cadherin phenotype where the *MGAT5* knockout resembles the M1 mutant and the M234+siGnT-V, whereas the GnT-V overexpressing mice are parallel with the M234 or M4 mutant. Furthermore and importantly from the clinical standpoint, we showed that gastric cancer patients with poor prognosis displayed a marked increased modification of E-cadherin with β1,6-branching *N*-glycans highlighting the importance of this specific E-cadherin aberrant glycoform in the gastric carcinogenic process.

Overall, this is the first study to clearly demonstrate that, among the four putative *N*-glycosylation sites, site 1 (Asn-554) in the EC4 domain of E-cadherin is the one that is selectively modified by the GnT-V glycosyltransferase in gastric carcinoma cells leading to its functional impairment. The molecular mechanism governing the specific biosynthesis of the β1,6 GlcNAc-branched structures at site 1 of E-cadherin, and not on any of the other three sites, remains unknown. Nevertheless, it is possible that, because this *N*-glycosylation site is the most extracellular (the furthest from the membrane), it is therefore more likely to regulate adhesion with neighboring cells. Additionally, the pathological context of gastric tumor cells may also account for this selective remodeling of E-cadherin *N*-glycosylation site 1 by GnT-V, which probably occurs depending on the physiopathological context and in a tissue- and cell-specific manner.[19,37–39]

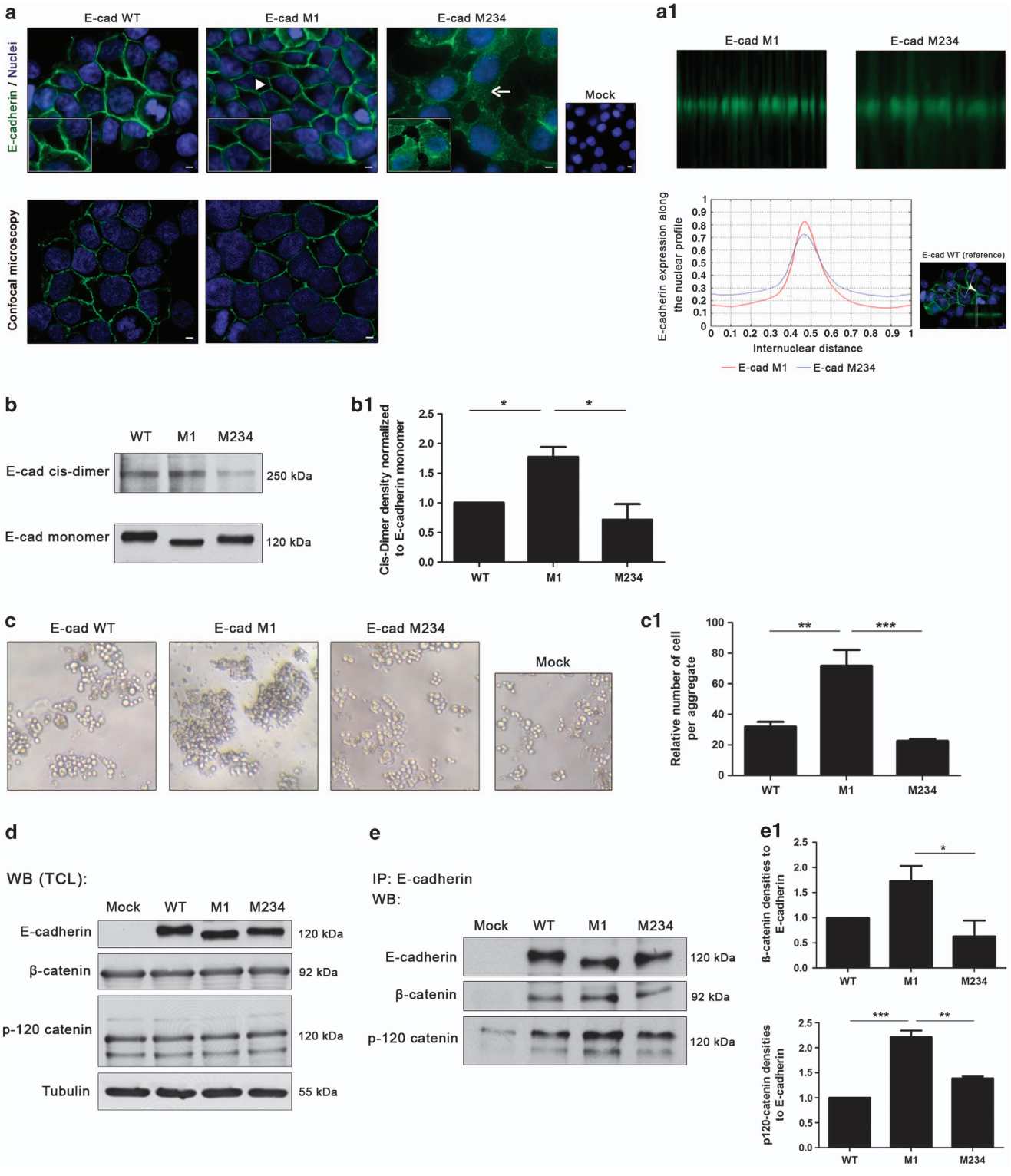
Taken together, we discover how site-specific E-cadherin glycosylation modification can directly modulate E-cadherin-mediated cell–cell adhesion, a key pathophysiological event in

cancer progression. Moreover, preventing E-cadherin site-specific glycosylation modifications was shown to improve its tumor-suppressive functions in gastric cancer. These results may open new avenues for the discovery of a promising (glyco)biomarker for improving early diagnosis, clinical surveillance and patients' risk stratification, as well as for the development of targeted-specific therapeutic strategies applied to the clinical setting.

MATERIALS AND METHODS

Tissue immunohistochemistry

Formalin-fixed paraffin-embedded tissue from normal, MGAT5 knockout (C57BL/6 background; generously provided by Professor Michael Pierce) and MGAT5 transgenic mouse stomach (C57BL/6 background; generously provided by Professor Eiji Miyoshi) were used for E-cadherin and L-PHA staining as previously described.[15]



In situ PLA

A series of human stomach samples from both normal mucosa ($n = 4$) and gastric carcinoma diffuse-type ($n = 19$) were obtained from the S. João Centre Hospital, Porto, Portugal and subjected to *in situ* PLA.[27] This study was approved by the committee of S.João Centre Hospital. An informed consent was obtained for all the following subjects.

An antibody against E-cadherin (Mouse anti-E-cadherin, clone 4A2C7) and L-PHA biotinylated lectin against the β1,6 GlcNAc-branched *N*-glycan structure (Vector Laboratories, Burlingame, CA, USA) were used. The PLA probes used were, respectively, anti-mouse immunoglobulins PLUS and anti-biotin-MINUS, conjugated to oligonucleotides. Images were acquired using brightfield and/or fluorescence microscope. The PLA signal was evaluated by three independent observers and scored as follows: negative, when absent/low expression of PLA signal was observed; and positive, when moderate/high expression of PLA signal was observed.

Prediction of N-glycosylation site occupancy

Amino-acid sequences of E-cadherin from various species are extracted from NCBI Reference Sequences[40] and selected by means of BLAST search.[41] Multiple top-hit sequences are aligned using Clustal W.[42] 3D homology modeling of E-cadherin was performed using the MODELLER version 9.8 software (San Francisco, CA, USA).[43] The crystal structures of the N-terminal domain of human E-cadherin (PDB ID 1o6s) and C-cadherin ectodomain (PDB ID 1l3w) were used as templates. The solvent ASA of the model was calculated using the DSSP program (San Franscisco, CA, USA).[44] A sliding window method was used to calculate the averaged ASA (window size = 5), and the value was assigned to the central amino-acid residue. The β-turn propensity was predicted by the Chou Fasman method[45] using updated PDB data.[46]

Plasmids construction and site-directed mutagenesis

A plasmid pcDNA3-E-cad containing human full-length E-cadherin cDNA (GenBank accession no. Z13009) was used to produce the E-cadherin *N*-glycan site mutations singly and in different combinations. PCR-based site-directed mutagenesis was carried out using the QuickChange XL Site-directed Mutagenesis Kit (Stratagene, Santa Clara, CA, USA). The mutagenesis primers used to the replacement of asparagine for glutamine in the potential *N*-glycosylation sites (Asn-554, Asn-566, Asn-618 and Asn-633) were created according to the manufacturer's instructions. All mutations were confirmed by DNA sequencing.

Release of N- and O-glycans and nanoPGC LC-ESI-MS/MS analysis

The *N*-glycans of immunoprecipitated E-cadherin WT and the respective mutants were released from PVDF membranes after SDS–PAGE separation and electro-blotting onto PVDF membranes to immobilize glycoproteins as described previously.[34,35] The released *N*-glycans were dissolved in 10 µl of pure water, and 5 µl were injected and analyzed by PGC nanoLC-ESI-MS/MS (Ultimate 3000 UHPLC system, Dionex, Sunnyvale, CA, USA) coupled to an amaZon ETD Speed ion trap equipped with a CaptiveSpray ionization source in negative ion mode (all Bruker Daltonics, Bremen, Germany). The instrument was set up to perform collision-induced dissociation fragmentation. An *m/z* range from 380 to 1850 Da was used for data-dependent precursor scanning with the SPS target mass set to 900 *m/z*. The capillary voltage was set at 1.3 kV, and the three most intense signals were selected for collision-induced dissociation experiments (MS/MS scan range 50–2500 *m/z*). MS as well as MS/MS data were recorded in the instrument's 'ultra scan mode'. Glycans were loaded onto a PGC precolumn (Hypercarb KAPPA 30 × 0.32 mm, 5 µm particle size) and separated on an analytical PGC column (Hypercarb PGC Column, Thermo, 100 mm × 75 µm, 3 µm particle size, both Thermo Fisher, Waltham, MA, USA). The samples were loaded onto the precolumn at a flow rate of 6 µl/min in 100% buffer A (10 mM ammonium bicarbonate). The starting conditions for the analytical column were 2% buffer B (10 mM ammonium bicarbonate in 60% acetonitrile) at a flow rate of 0.8 µl/min. The gradient conditions were as follows: increase of buffer B from 3% to 15.8% (6 min), further increase to 40.3% B (6–55 min), followed by a steeper increase to 90% B (55–60 min). The column was held at 90% B for 6 min. At the same time, the precolumn was flushed with 90% Buffer C (10 mM ammonium bicarbonate in 90% acetonitrile) at a flow rate of 6 µl/min before re-equilibrating the precolumn as well as the analytical column in 98% buffer A for 5 min. Glycans were automatically identified using an in-house established *N*-glycan spectral database and the spectral library tool integrated in Compass Data Analysis 4.1 (Bruker Daltonics). *N*-glycan structures not present in the database were assigned manually if sufficient data could be acquired. If no unambiguous identification of a structure was possible based on the MS/MS and LC data but just on MS data only, the respective portion of the *N*-glycan is labeled accordingly in the Supplementary Tables S1 and S2. Glycan compositions were determined using the GlycoMod tool available on the ExPASy server[47] (http://web.expasy.org/glycomod/; with a mass tolerance of 0.3 Da in the negative ion mode, reduced *N*-glycan oligosaccharides, respectively, and restricted to the monosaccharide residues hexose (Hex), *N*-acetyl hexosamine (HexNAc), deoxyhexose, *N*-acetyl neuraminic acid (NeuAc), *N*-glycolyl neuraminic acid (NeuGc) and sulfate.

The accessed glycan structures were then manually validated from tandem MS fragmentation spectra using the Glycoworkbench software tool (Eurocarb, Cambridge, UK).[48] Data reporting has been performed as proposed by the MIRAGE guidelines.[49,50]

Data processing and relative quantitation of PGC glycomics data

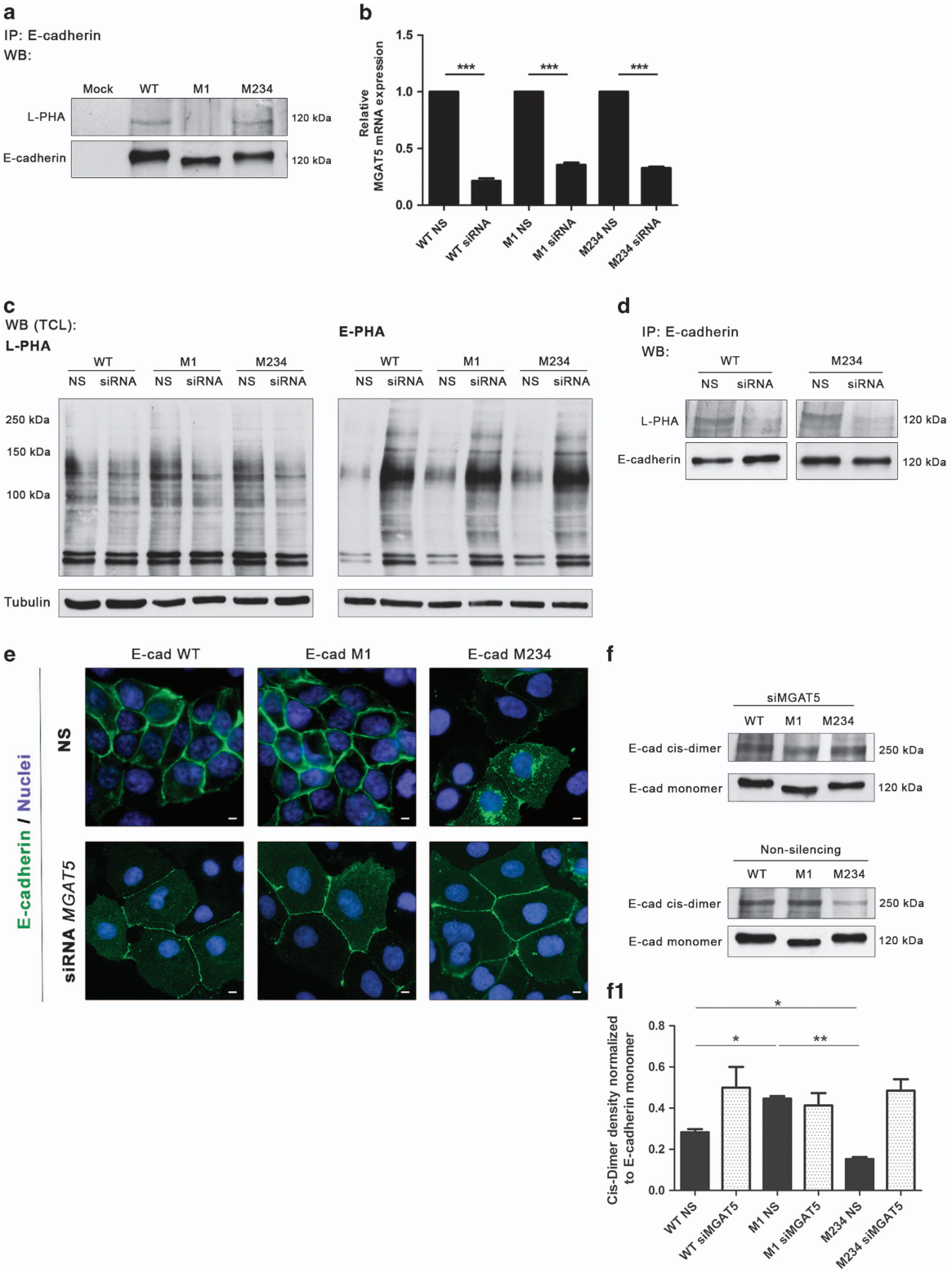
The peak area of each glycan composition and isoform was calculated using the respective extracted ion chromatograms using Compass QuantAnalysis (Bruker Daltonics). The integration of every extracted ion chromatogram was validated manually, in particular for peaks with very low abundance. The generated data were imported in CSV format, converted in Excel (Microsoft Office Excel 2013, Redmond, WA, USA) and expressed as a relative abundance value for each individual *N*-glycan

Figure 4. *N*-glycosylation at Asn-554 affects E-cadherin biological functions. (**a**) Immunofluorescence analysis showing that AGS cells expressing E-cadherin WT displayed an epithelial morphology and a membrane localization of E-cadherin (arrowhead). E-cadherin M1 showed a correct localization of E-cadherin at the cell membrane (arrowhead) with a more focused membrane staining than the WT (confocal microscopy). *N*-glycan mutant with Asn-554 (site 1) occupied with *N*-glycan structures (M234) is characterized by an incomplete localization of E-cadherin at the cell–cell contacts together with some cytoplasmic staining (arrow). White size bars ~5 µm. (**a1**) Evaluation of the internuclear profiles of E-cadherin M1 and E-cadherin M234. Mutation at site 1 (E-cadherin M1) induces an increased membrane localization of E-cadherin compared with M234, which shows a decreased E-cadherin membrane expression. (**b**) Evaluation of *cis*-dimer-formation capacity of E-cadherin using BS^3. Mutation of Asn-554 (M1) leads to a significant increase of *cis*-dimerization of E-cadherin. (**b1**) Bar graphs. Amounts of E-cadherin *cis*-dimer were determined from the ratio of densities of E-cadherin *cis*-dimer/E-cadherin monomer. Results are described as mean ± s.d. of three independent experiments. The E-cadherin *cis*-dimer formation in E-cadherin M1 and M234 are expressed as the fold increase, compared with the E-cadherin WT, which was taken as 1 (Student's *t*-test: $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$). (**c**) *In vitro* cell–cell aggregation assay. Mutation at Asn-554 (M1) resulted in a significant increase of cell–cell aggregation than E-cadherin WT and M234 showing larger cell aggregates. (**c1**) Bar graphs. Quantification of cell–cell aggregation by measuring the relative number of cells per aggregate (three or more cells). Results are described as the mean ± s.d. of three independent experiments. Student's *t*-test: $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$). (**d**) Evaluation of the β- and p120-catenin total protein expression levels in the total protein lysates from AGS Mock, AGS E-cadherin WT and E-cadherin *N*-glycan mutants M1 and M234 cells. No significant differences were observed among the E-cadherin WT and E-cadherin *N*-glycan mutants. (**e**) E-cadherin immunoprecipitation followed by β- and p120-catenin Western-blot. The results showed that mutation of site 1 (Asn-554), M1, is associated with an increased interaction between E-cadherin and the β- and p120-catenin. A decreased interaction between E-cadherin and β-catenin (of about 1.0-fold) in M234 mutant compared with E-cadherin M1 was also verified. (**e1**) Bar graphs. Amounts of association were determined from the ratios of densities of β- or p120-catenin after normalization to E-cadherin. Results are described as mean ± s.d. of three independent experiments. The E-cadherin/catenin interaction levels in E-cadherin M1 and M234 are expressed as the fold increase, compared with E-cadherin WT, which was considered as 1 (Student's *t*-test: $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$). See also Supplementary Figure S4.

structure, respectively, within each E-cadherin type. The *N*-glycans were classified into three categories (high mannose, neutral or sialylated).

Cell culture and transfection

MDCK (Madin Darby canine kidney), BT20 (breast tumor cell line), HT29 (human colon cancer cell line) and AGS cells (human adenocarcinoma epithelial cell line) were grown in monolayer culture and maintained at 37 °C in an atmosphere of 5% CO_2 in RPMI 1640 GlutaMAX (or Dulbecco's modified Eagle's medium for BT20 cells), HEPES medium (Invitrogen, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco, Invitrogen) and 1% penicillin–streptomycin (Gibco, Invitrogen). These cells were obtained from American Type Culture Collection (Manassas, VA, USA), which were tested and authenticated by the cell bank using their



standard short tandem repeats–based techniques. Cells were also monitored by microscopy to maintain their original morphology and also tested for mycoplasma contamination.

Cells were transfected at 40–50% confluence with E-cadherin WT and E-cadherin *N*-glycan mutants using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

Westerns blotting, lectin blotting and immunoprecipitation

Cell protein lysates from different E-cadherin *N*-glycan mutants were subjected to 7.5% SDS–PAGE electrophoresis, transferred to nitrocellulose membranes and probed with the primary antibody against E-cadherin, β-catenin, p120-catenin and tubulin, as previously described.[15]

The expression of β1,6 GlcNAc-branched *N*-glycan structures and the bisecting GlcNAc *N*-glycans were detected by lectin blotting analysis, where membranes were incubated, respectively, with L-PHA (that specifically recognizes β1,6 GlcNAc-branched *N*-glycans, product of GnT-V) and E-PHA (that specifically recognizes bisecting GlcNAc *N*-glycans, product of GnT-III) lectins (1 μg/ml; Vector Laboratories).[15] For the β1,6 GlcNAc-branched *N*-glycans analysis on E-cadherin immunoprecipitated, equal amounts of total protein (750–1500 μg) from each cell lysate were used and the membranes were probed with L-PHA lectin.[15]

PNGase F and *Endo H* digestion

Total cell lysates (30 μg) were combined with denaturating buffer and incubated at 100 °C for 10 min. Samples were then digested for 3 h with 1 unit of *PNGase F* or *Endo H* (New England Biolabs, Hertfordshire, UK) at 37 °C. *Endo H* is an endoglycosidase that cleaves within the chitobiose core of high mannose and some hybrid oligosaccharide structures in *N*-linked glycoproteins. *PNGase F* is an amidase that cleaves between the innermost GlcNAc and asparagine residues of high-mannose, hybrid and complex oligosaccharides from *N*-linked glycoproteins. The deglycosylated proteins were loaded onto 7.5% SDS–PAGE and immunoblotting with anti-E-cadherin. For controls, the samples were incubated without the enzymes.

Calcium switch

AGS expressing E-cadherin WT, M1 and M234 were washed with phosphate-buffered saline (PBS), incubated with EGTA 2 mM for 10 min and supplemented with medium. Cells were then lysed after 2, 4 and 24 h

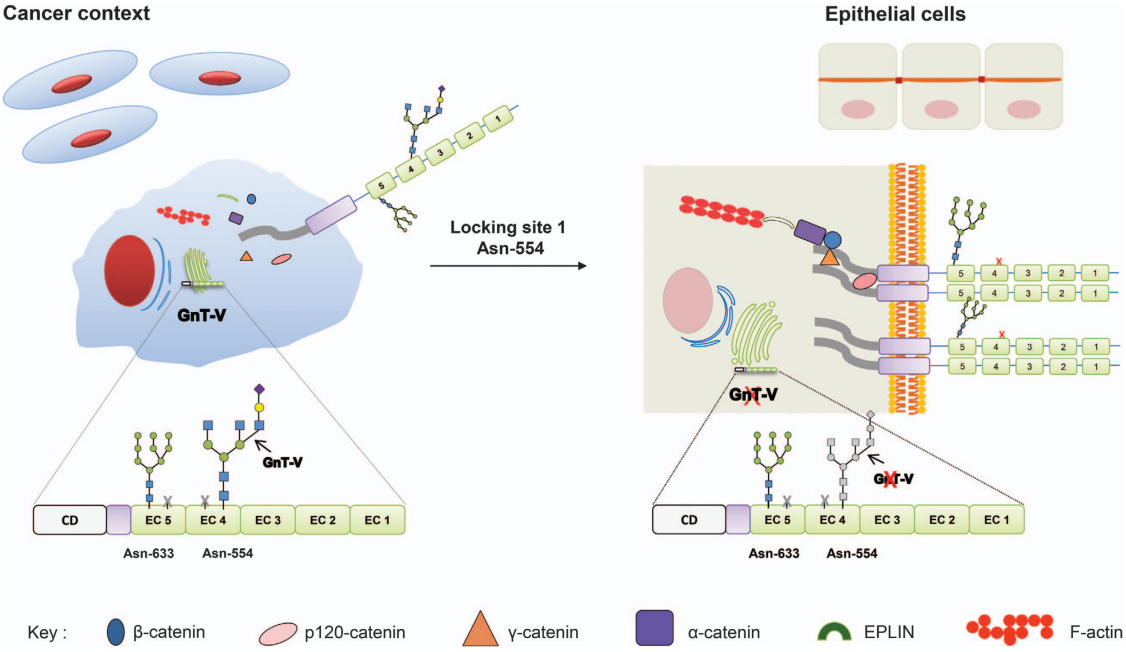


Figure 6. Proposing model for the site-specific E-cadherin *N*-glycosylation in cancer context. The site-specific occupancy of E-cadherin Asn-554 with β1, 6 GlcNAc-branched structures catalyzed by GnT-V impairs its biological functions leading to a decreased E-cadherin *cis*-dimerization capability, a decreased cellular aggregation and an impairment of the molecular assembly of adherens junctions. Locking this site-specific modification either by Asn-554 mutation or by GnT-V silencing recovers the biological functions of E-cadherin by increasing E-cadherin cis-dimerization, aggregation and the stability of the E-cadherin–catenin complex.

Figure 5. Knockdown of GnT-V induces the recovery of E-cadherin expression and functions. (**a**) Immunoprecipitation of E-cadherin M234 showed an increased L-PHA reactivity compared with the M1 mutant, revealing a significant increase in β1,6 GlcNAc-branched *N*-glycan structures modification in comparison with E-cadherin M1. (**b**) *MGAT5* mRNA expression of AGS cells expressing E-cadherin WT, M1 and M234 after *MGAT5* silencing. Around 70–80% of *MGAT5* silencing was observed in WT, M1 and M234 mutants. NS, non-silencing. The relative mRNA expression of siRNA cells expressing E-cadherin WT, M1 and M234 are expressed as the fold increase, compared with the respective non-silencing cells, which was taken as 1 (Student's *t*-test: $*P \leqslant 0.05$; $**P \leqslant 0.01$; $***P \leqslant 0.001$). (**c**) Lectin blot analysis of GnT-V and GnT-III products on total cell lysate from AGS cells expressing E-cadherin WT, M1 and M234 after *MGAT5* knockdown. A decreased L-PHA reactivity after *MGAT5* knockdown and a competitive increase of E-PHA reactivity were observed. (**d**) *MGAT5* silencing resulted in a remarkable decreased L-PHA reactivity for the E-cadherin band from the E-cadherin WT and E-cadherin M234, suggesting that Asn-554 is occupied with β1,6 GlcNAc-branched *N*-glycans. (**e**) Immunofluorescence analysis of AGS cells expressing E-cadherin WT, M1 and M234 after *MGAT5* knockdown. After *MGAT5* silencing, E-cadherin M234 showed an increased localization at the cell–cell membrane. White size bars ~5 μm. (**f**) Evaluation of the impact of *MGAT5* silencing on *cis*-dimer formation of E-cadherin. Knockdown of *MGAT5* leads to a similar ratio of E-cadherin *cis*-dimer formation in AGS cells expressing E-cadherin M1 and M234. (**f1**) Bar graphs. Amounts of E-cadherin *cis*-dimer were determined from the ratio of densities of E-cadherin *cis*-dimer/E-cadherin monomer. Results are reported as the mean ± s.d. of two independent experiment. See also Supplementary Figure S6.

with buffer containing PBS, 1% Triton X-100 and 1% NP40, and 30 μg of total cell lysate were subjected to 7.5% SDS–PAGE.

E-cadherin cis-dimer formation

Cells were plated in six-well plates to confluence. They were washed with PBS and incubated with 2.5 mM BS3 (Sigma, St Louis, MO, USA) for 3 h on ice. The resulting cells were then incubated with 10 mM Tris (pH 7.5) for 15 min on ice. Cells were then lysed with buffer containing PBS, 1% Triton X-100 and 1% NP40, and 30 μg of total cell lysate were subjected to 6% SDS–PAGE. E-cadherin *cis*-dimer formation was detected by immunoblotting.

Immunofluorescence

AGS cells transfected with the different E-cadherin *N*-glycans mutants were plated on six-well plates with coverslips. Cells were fixed with methanol and blocked with bovine serum albumin 5% in PBS. Cells were incubated with mouse anti-E-cadherin monoclonal antibody.[15] Immunofluorescent images were obtained using a Zeiss Imager.Z1 AxioCam MRm (Carl Zeiss, Jena, Germany).

For the quantitative analyses of *in situ* immunofluorescence images, a 1D (one dimension) representative profile of protein level of expression and distribution was obtained. To undertake this analysis, original immunofluorescence images from cell populations were used to extract internuclear profile map from single cells and pairs of cells.[51]

Slow-aggregation assay

AGS cells transfected with E-cadherin WT and the different E-cadherin *N*-glycan mutants (5×10^4) were seeded onto an agar gel (0.66% w/v) in a 96-well plate. Aggregation formation was evaluated after 24 h using inverted phase-contrast microscope. Experiments were carried out in triplicate.

siRNA transfection

siRNA targeting *MGAT5* and control siRNA were purchased from Dharmacon (Lafayette, CO, USA). si*MGAT5* (0–200 nM) was transfected into AGS cells expressing Mock, E-cadherin WT, M1 and M234 with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Efficiency of *MGAT5* knockdown was optimal with 80 nM of siRNA after 48 h.

Statistical analysis

PLA analyses were performed using the SPSS statistical software (IBM, Armonk, NY, USA). Statistical associations between clinicopathological features and aberrant E-cadherin *N*-glycosylation mediated by GnT-V were assessed using the χ^2 test. Survival curves were estimated using the Kaplan–Meier method.

Statistical analyses were performed using the Graph Pad program (GraphPad Software, Inc., La Jolla, CA, USA). Student's tests were used to calculate significance in an interval of 95% confidence level. All statistics were compared with the E-cadherin WT group, and values of $P < 0.05$ were considered to be statistically significant (Student's *t*-test: $^*P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Chapter IV

O-mannosylation and N-glycosylation: two coordinated mechanisms regulating the critical functions of E-cadherin in cancer

O-Mannosylation and N-glycosylation: two coordinated mechanisms regulating the critical functions of E-cadherin in cancer.

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Running Title

Deficiency on E-cadherin O-mannosylation in cancer.

Abstract

E-cadherin plays a major role in malignant cell transformation and impairment of its biological functions is a well-established molecular event occurring during gastric carcinogenesis. Glycosylation modifications were identified as a fundamental mechanism of E-cadherin dysregulation in cancer with promising clinical applications. Nevertheless, it remains to be determined the role of the recently identified O-mannosyl glycans in the regulation of E-cadherin functions in cancer and how this novel post-translational modification interplay with E-cadherin N-glycans in a cancer context. In this study we found that the overall O-mannosylation profile of cancer proteins is reduced in gastric cancer patients comparing with normal gastric mucosa. Moreover, and concomitantly with the acquisition of a poorly-differentiated gastric cancer phenotype, E-cadherin suffers a decrease on the expression of O-mannosyl glycans with effects on the impairment of its biological functions. Interestingly, when the O-mannosylation pathway was promoted through protein O-mannosyltransferase 2 (POMT2) overexpression, a recovery of E-cadherin localization at cell-cell borders together with an improvement on the adherens junctions assembly and competence were observed. We further demonstrated that O-mannosylation regulates the critical functions of E-cadherin in cancer in coordination with N-glycosylation. Genetic manipulation of the O-mannosylation pathway, either by POMT2 overexpression or silencing, resulted in alterations in the expression of complex branched N-glycans modulating E-cadherin functions. Likely, overexpression of branched N-glycans, led to a significant decrease of O-mannosyl glycans on E-cadherin. This mechanistic interplay between O-mannosyl glycans and branched N-glycans was further demonstrated to occur at E-cadherin site-specific level. Preventing E-cadherin Asn-554 from receiving the deleterious branched structures, resulted in an increased O-mannosylation profile of E-cadherin that culminated in a protective effect on the glycoprotein by precluding its functional dysregulation and contributing to tumor suppression. These results were further confirmed using Mgat5 transgenic mouse models and also in human gastric carcinomas.

Introduction

Epithelial cadherin (E-cadherin) is a cell surface glycoprotein with key roles in normal homeostasis, through the establishment and maintenance of competent intercellular adhesions, cell polarity and normal tissue architecture (1-4). E-cadherin molecule establishes homophilic interactions through its ectodomains establishing strong adhesive complexes (5, 6). Molecular interactions of the E-cadherin cytoplasmic domain with cytosolic proteins, called catenins, also provides anchorage to the actin cytoskeleton contributing to the establishment of stable and mature adherens junctions (4, 7).

E-cadherin plays a major role in malignant cell transformation. Reduced levels of E-cadherin expression and impairment of its functions have been reported to be a well-established molecular event that occurs during tumour development and progression, leading to an increased ability of cells to invade neighbouring tissues and to metastasize (8-10).

Posttranslational modifications of E-cadherin through glycosylation have been pinpointed to play an instrumental role in the dysregulation of E-cadherin functions in a cancer context. (11-13). Indeed, altered pattern of protein glycosylation originates different E-cadherin glycoforms that have been associated with the impairment of its critical functions in gastric cancer (14).

Modifications of E-cadherin with β1,6 GlcNAc-branched N-glycans, catalysed by the N-acetylglucosaminyltransferase V (GnT-V), has major deleterious effects on E-cadherin-mediated cell-cell adhesion leading to the impairment of the stability and competence of the intercellular adhesive complex (14-16). The importance of this specific E-cadherin aberrant N-glycoform was demonstrated to be associated with invasive and metastatic potential of tumour cells and thus with the pathogenesis of gastric carcinoma (17). Moreover, it was recently reported by us that among the four potential N-glycosylation sites on the extracellular domain of E-cadherin, the Asn-554 is the key site that, within a gastric cancer context, is selectively modified with the deleterious β1,6 GlcNAc-branched N-glycans catalysed by Gnt-V, directly affecting E-cadherin functions (17). Interestingly from the clinical standpoint, prevention of E-cadherin aberrant glycosylation at Asn-554 was found to preclude its functional dysregulation by improving its critical function in gastric cancer (17).

Beyond N-glycosylation, E-cadherin was recently identified as a major target of O-mannosylation (18, 19). Protein O-mannosylation is a posttranslational process that is initiated at the endoplasmic reticulum (ER) by the covalent attachment of mannose structures to serine (Ser) or threonine (Thr) residues of secretory and/or membrane proteins catalysed by the homologous protein O-mannosyltransferase 1 (POMT1) and 2 (POMT2)

(20, 21). These O-mannose core structures may be further extended via different linkages originating distinct extended O-mannosylated structures (22). According to the literature, the most well characterized O-mannosylated mammalian protein is α-dystroglycan (α-DG), an integral glycoprotein of the dystrophin-glycoprotein complex. The α-DG links the extracellular matrix (ECM) to the actin cytoskeleton by interacting with ECM proteins in a glycosylation-dependent manner. Disruption of the O-mannosylation pathway causing the hypoglycosylation of α-DG results in the impairment of α-DG-mediated epithelial cell-basement membrane interaction, and underlies various forms of congenital muscular dystrophies (CMD) (23-25). A wide spectrum of O-mannosylated proteins were recently identified, and among them, cadherins revealed as major O-mannosylated glycoproteins (18, 19, 26). Lommel et al. demonstrated that O-mannosylation of E-cadherin is required for the morula to blastocyst transition before implantation. Moreover, O-mannosylation was found to be important for E-cadherin-mediated cell-cell adhesion in a normal context (18). Absence of O-mannosylated structures at the EC2-5 domains leaded to E-cadherin abnormal localization (19). Taken in consideration the instrumental role of glycosylation as a mechanism for regulating E-cadherin functions in cancer (11, 12), it is of utmost importance to unravel the yet uncover molecular role of O-mannosyl glycans and their interplay with N-glycans in the regulation of E-cadherin biological functions in cancer.

In this study, we report that O-mannosylation is overall reduced in gastric cancer compared with normal gastric mucosa. Particularly, E-cadherin suffers a decrease in the O-mannosylation profile concomitantly with the acquisition of a poorly-differentiated gastric cancer phenotype. This deficiency on O-mannosylated glycans on E-cadherin compromises the stability of the adherens junctions associated with tumour progression. Furthermore, we also demonstrate the existence of a coordinated interplay between complex N-glycans and O-mannosyl glycans on E-cadherin with biological relevance in a cancer context. Interestingly, prevention of the addition of β1,6 GlcNAc-branched N-glycans at the Asn-554 of E-cadherin promotes the modification with O-mannosyl glycans, leading to a recovery of E-cadherin functions. Further, this interplay between branched N-glycans and O-mannosyl glycans were observed both in transgenic animal models and *in* human diffuse gastric cancer patients.

Material and Methods

Tissue immunohistochemistry and lectin staining

Formalin-fixed paraffin-embedded tissue from normal human gastric mucosa, human diffuse gastric cancer (n=3; both obtained from the S. João Centre Hospital, Porto, Portugal); MGAT5 knockout and MGAT5 transgenic mouse stomach (C57BL/6 background; generously provided by Professor Michael Pierce and Eiji Miyoshi, respectively) were used for E-cadherin and L-PHA staining as previously described (14). For O-mannosylated proteins staining, the sections were incubated with T[α1- Man]- specific polyclonal antibody (dilution 1:50) for one hour at RT. This study was approved by the ethical committee of Centro S. João Centre Hospital, Porto, Portugal and an informed consent was obtained for all the subjects.

Cell lines

Human gastric carcinoma cell lines MKN28, Kato III, and AGS cells transfected with E-cadherin WT and E-cadherin N-glycan mutants, as previously described (17), were grown in RPMI 1640 GlutaMAX supplemented with 10% fetal bovine serum (Gibco, Invitrogen) and 1% penicillin-streptomycin (Gibco, Invitrogen), and maintained at 37 ºC in an atmosphere of 5% CO_2. MKN45 gastric cell line stably transfected with GnT-V and with the empty vector (mock cells) were cultured in the previous conditions under the selection of G418 (500 µg/mL) (27-29).

These cells were obtained from American Type Culture Collection (Manassas, VA, USA), which were tested and authenticated by the cell bank using their standard short tandem repeats-based techniques. Cells were also monitored by microscopy to maintain their original morphology and also tested for mycoplasma contamination.

Cell transfection and siRNA transfection

Kato III suspension cells were transfected with plasmid pTW49 using Lipofectamine 2000 (Invitrogen), according to the manufacturer´s instructions. siRNA targeting POMT2 (ON-TARGETplus Human POMT2siRNA-SMARTpool) and control siRNA (ON-TARGETplus Non-targeting pool) were purchased from Dharmacon. siPOMT2 (0-200 nM) was transfected into MKN28 cell line with Lipofectamine 2000 (Invitrogen) according to the manufacturer´s instructions. Efficiency of POMT2 knockdown was optimal with 80 nM of siRNA after 48h.

RNA expression and quantification

Total RNA was extracted from cell lines using TRI reagent (Sigma-Aldrich), according to manufacturer´s protocol. RNA yield and quality were determined spectophotometrically and 1000 ng of total RNA were reverse transcribed to single stranded cDNA using Superscript II Reverse Transcriptase and random hexamer primers (Invitrogen, Oregon, USA). Quantitative Real-Time PCR was carried out in triplicates using source RNA from three biological replicas, for the target genes POMT2, POMT1 and MGAT5 and for the endogenous control 18S. Data was analyzed by the ΔΔCT method (30). Statistical analysis was performed using GraphPad PRISM 5.0 software and significance was evaluated with Student´ s T-Test.

Cell and Tissue Lysate Preparation

Fresh tissue from normal human gastric mucosa and diffuse gastric cancer were obtained from the S. João Centre Hospital, Porto, Portugal. Tissues were dissected into smaller pieces, immersed in liquid nitrogen to snap freeze, and stored at -80ºC for later use. To proceed to lysate preparation, tissues were washed briefly with PBS1x to remove any blood, and homogenized with an electric homogenizer with ice cold NP-40 lysis buffer containing protease inhibitors (for ~ 5 mg piece of tissue, ~300 µL of lysis buffer was added). To prepare lysate from cell culture, the cell culture dish were washed with ice-cold PBS, and the adherent cells were scraped off with ice cold NP-40 lysis buffer. Regarding Kato III suspension cells, cell suspension from culture dish was transferred into tube, washed with ice-cold PBS and homogenized with ice cold NP-40 lysis buffer. The samples were maintained on ice during 20 minutes, then centrifuged at 13200 rpm for 20 minutes at 4ºC, and the supernatant was transferred to a fresh tube. Total protein was quantified using a BCA protein assay kit (Pierce).

Immunoblotting, lectin blotting

Protein lysates were subjected to 7.5% SDS-PAGE electrophoresis, transferred to nitrocellulose membranes and probed with the primary antibody against E-cadherin and β-actin/ α-tubulin, as previously described (14). POMT2 expression was evaluated incubating the membranes with polyclonal rabbit anti-POMT2 (1:500).

The expression of β1,6 GlcNAc-branched N-glycans structures were detected by lectin blotting, where membranes were incubated with L-PHA lectin (1-5 µg/mL; Vector Laboratories). Expression of O-Mannosyl glycans was evaluated by *PNGase F* digestion to remove N-glyans (overnight, 37ºC) followed by lectin blotting using Biotinylated Concanavalin A (Con A) (1-5 µg/mL; Vector Laboratories). For the β1,6 GlcNAc-branched N-glycans analysis on E-cadherin immunoprecipitated, equal amounts of total protein (500-

1000 µg) from each cell lysate were used and the membranes were probed with L-PHA lectin. Regarding the evaluation of O-Man glycans on E-cadherin, E-cadherin immunoprecipitated was subject to *PNGase F* digestion overnight at 37ºC, and the membranes were probed with Con A lectin.

Immunofluorescence

Cells were grown on six-well plates with coverslips. Cells were fixed with methanol and blocked with bovine serum albumin (BSA) 5% in PBS. Cells were then incubated with mouse anti-E-cadherin monoclonal antibody (BD Bioscience; 1:200 diluted in BSA 5%) for one hour at RT. After primary antibody incubation, the cover slips were washed in PBS and then Alexa Fluor 488 anti-mouse was used as secondary antibody (Invitrogen; 1:500 diluted in BSA 5%; one hour of incubation in the dark). Finally, the cover slips were washed in PBS and mounted on slides using Vectashield with DAPI (Vector Laboratories). Immunofluorescent images were obtained using a Zeiss Imager.Z1 AxioCam MRm (Carl Zeiss, Jena, Germany).

Slow-aggregation assay

Wells of a 96-well-plate were coated with 50 µl of an agar solution. Cells ($1,25 \times 10^4$) were seeded onto an agar gel (0.66% w/v) in a 96-well plate. Experiments were carried out in triplicate.

Statistical analysis

Statistical analyses were performed using the Graph Pad program (GrapPad Software, Inc., La Jola, CA, USA). Student´s tests were used to calculate the significance in an interval of 95% confidence levels. Values of $P < 0,05$ were considered to be statistically significant (Student´s t-test: $*P \leq 0,05$; $**P \leq 0,01$; $***P \leq 0,001$).

Results

Protein O-Mannosylation is reduced in human gastric carcinoma

In order to assess the significance of protein O-mannosylation in a cancer context, we evaluated the overall O-mannosylation profile in normal gastric mucosa and in diffuse gastric cancer, using the T[α1-Man]-specific polyclonal antibody. This antibody was generated to direct against a threonine O-mannosyl-conjugated epitope and specifically detects O-mannosylated proteins (18). Normal gastric mucosa (from the body region of the stomach), displaying a normal basolateral cell membrane localization of E-cadherin, exhibited a strong reactivity of O-mannosylated proteins at the cytoplasm and/or at the cell membrane (Figure 1A). Conversely, in diffuse gastric cancer cases showing an abnormal/aberrant pattern of E-cadherin expression, a significant decrease of O-mannosylated proteins was observed. Interestingly, O-mannosylated proteins were detected in normal adjacent mucosa, predominantly in cells displaying a normal pattern of E-cadherin membrane localization.

Afterwards, we evaluated the expression levels of POMT2 protein and O-mannosyl glycans *in vivo*, in gastric carcinomas (Figure 1B). As expected, the total E-cadherin expression is decreased in diffuse gastric cancer comparing with normal gastric mucosa. Likewise, the total levels of POMT2 protein expression are reduced in diffuse gastric carcinoma, comparing with normal mucosa. Further, the O-mannosyl glycans profile was evaluated by removal of N-glycans with PNGase F treatment followed by Concanavalin A (Con A) lectin blot, which thereby recognizes O-Man glycoproteins. Figure 1B showed that O-mannosylation profile decreased in diffuse gastric carcinoma (see also Supplementary Figure 1).

These observations suggest that human normal gastric mucosa, particularly from the body region of the stomach, is characterized by a high expression of O-mannosylated proteins that are decreased in human diffuse gastric carcinoma.

O-mannosylation of E-cadherin decreases in poorly-differentiated gastric cancer phenotype.

To further evaluate the role of O-mannosylation in cancer, we investigated the expression of O-mannosyl glycans in two distinct gastric cancer cell lines that represent different stage of gastric cancer differentiation, behavior and progression: the MKN28 gastric cancer cell line, which is a well differentiated tubular adenocarcinoma with normal

E-cadherin functions, and the Kato III gastric cancer cell line, which is an undifferentiated gastric signet ring cell line with abnormal E-cadherin expression and functions (31-33).

MKN28 gastric cancer cells exhibited an epithelial morphology forming larger compact cellular aggregates, whereas Kato III cell line is characterized by sparse cells displaying some fibroblatoid-like appearance with reduced cell-cell aggregation capability (Figure 2A). Regarding the E-cadherin cellular localization, the immunofluorescence staining revealed that MKN28 cells showed a strong membranous staining of E-cadherin, whereas the expression of E-cadherin in Kato III cells is reduced at the cell-cell contacts with mislocalization into the cytoplasm.

Concerning the O-mannosylation profile, a significant decrease in the total protein expression of POMT2 was observed in Kato III cells (Figure 2B). Accordingly, this undifferentiated gastric cancer cell line exhibited lower levels of O-mannosyl glycans expression (revealed after removal of N-glycans by *PNGase F* digestion), when compared with the well-differentiated MKN28 cells (Figure 2B, Supplementary Figure 2A). Concerning the mRNA transcription levels of POMTs, lower levels of POMT2 mRNA transcription were observed in Kato III cell line when compared with MKN28 (Figure 2C). No significant differences were detected on POMT1 mRNA expression levels (Supplementary Figure 2B).

Having demonstrated a decreased POMT2 and O-mannosyl glycans expressions in undifferentiated gastric cancer cells, we further evaluated the levels of O-mannosyl glycans specifically on E-cadherin protein. Figure 2D demonstrates that E-cadherin from MKN28 cells showed a significant increased reactivity with the Con A- mannose binding lectin after release of N-glycans, when compared with E-cadherin from Kato III (see also Supplementary Figure 2C). These results suggest that E-cadherin is highly O-mannosylated in a well-differentiated cancer context and this modification decreases during the acquisition of an undifferentiated cancer phenotype.

Modulation of POMT2 expression impacts O-mannosylation profile of E-cadherin regulating its biological functions in cancer.

Having demonstrated the distinct O-mannosylation profiles of E-cadherin associated with different cancer phenotypes (Figure 2D), together with the observed differences on the levels of POMT2 gene transcription among the two cancer cells phenotypes (Figure 2C), we further evaluated the impact of O-mannosylation in the regulation of E-cadherin biological functions in a cancer context. For doing so, POMT2 expression was modulated either by silencing of *POMT2* gene in MKN28 cells (Figure 3) or by *POMT2* overexpression in Kato III cells (Figure 4).

The figure 3A showed a significant reduction of *POMT2* mRNA transcription levels after *POMT2* gene silencing, as well as a decrease in POMT2 protein expression (Figure 3B). Likely, the expression of overall O-mannosyl glycans was reduced after *POMT2* silencing (Figure 3B). Furthermore, knockdown of *POMT2* resulted in a decreased modification of E-cadherin with O-mannosyl glycans (Figure 3C, see also Supplementary Figure 3A). Consequently, by immunofluorescence analysis we verified an abnormal E-cadherin localization at the cell membrane and this mislocalization was accompanied with loss of cell-cell contacts (Figure 3D). In addition, *POMT2* knockdown resulted in a significant decreased interaction of E-cadherin with p120-catenin (Figure 3E). No significant differences were observed regarding the recruitment of β-catenin by E-cadherin upon *POMT2* silencing. Overall, these results indicated that the reduction of O-mannosylation of E-cadherin after *POMT2* silencing resulted in an impairment of E-cadherin cellular localization, disturbing the assembly of the adherens junctions through a decreased recruitment of catenins by E-cadherin.

To further evaluate the impact of O-mannosylation on E-cadherin functions, the opposite approach was performed by overexpressing *POMT2* gene in Kato III gastric cancer cells (Figure 4A). The results showed that *POMT2* overexpression resulted in an increased expression of POMT2 protein as well as an increase in overall O-mannosylated glycoproteins (Figure 4B). Looking specifically to E-cadherin glycoprotein, we observed that *POMT2* overexpression lead to an increased O-mannosylation of E-cadherin (Figure 4C, see also Supplementary Figure 3A). This increased modification of E-cadherin with O-mannosyl glycans was accompanied with a recovery of E-cadherin localization at the cell-cell borders (Figure 4D). Moreover, the recruitment of β-catenin and p-120-catenin by E-cadherin was also significantly improved after *POMT2* overexpression (Figure 4E).

These results reveal that O-mannosylation of E-cadherin is essential for its correct localization at the cell membrane playing also a crucial role in the molecular assembly and stability of the adherens junctions.

N-glycosylation and O-mannosylation as inter-players in the regulation of E-cadherin functions in cancer. The impact of site-specific N-glycosylation on E-cadherin O-mannosylation.

Our previous studies demonstrated that addition of GnT-V-mediated β1,6 GlcNAc-branched N-glycans on E-cadherin contributed to the abrogation of its critical functions in cancer (14, 17). Furthermore, recent studies reported that E-cadherin is also major target of O-mannosylation that appears to be important for cadherin-mediated

adhesive functions (18). In this study we evaluated the interplay between these two major forms of E-cadherin modification: GnT-V-mediated N-glycosylation and O-mannosylation and their role in the regulation of E-cadherin functions in gastric cancer.

The analysis of the levels of β1,6 GlcNAc-branched N-glycans (using L-PHA lectin) and O-mannosyl glycans (using Con A lectin after PNGaseF digestion) on E-cadherin from the two distinct gastric cancer phenotypes showed that in a well-differentiated cancer phenotype (MKN28), E-cadherin appears to be predominantly modified with O-mannosyl glycans than β1,6 GlcNAc branched N-glycans (Figure 5A). On the contrary, E-cadherin from a poorly-differentiated gastric cancer phenotype (Kato III) mainly exhibited β1,6 GlcNAc branched N-glycans. Interestingly, this interplay is also verified at the level of *POMT2* and *MGAT5* gene transcription, where *POMT2* gene is more expressed in MKN28 cells than in Kato III cells whereas MGAT5 gene is highly expressed in Kato III cells when compared with MKN28 cells (Supplementary Figure 4A).

Afterwards we went to assess the impact of POMT2 modulation (either by silencing or overexpression) in the levels of β1,6 GlcNAc-branched N-glycans attached to E-cadherin. The results showed that *POMT2* knockdown in MKN28 cells resulted in a significant increased expression of β1,6 GlcNAc branched N-glycans on E-cadherin (Figure 5B), although a slight decrease of *MGAT5* gene at the mRNA transcription level was verified (Supplementary Figure 4B). Conversely, the overexpression of *POMT2* gene in Kato III cells led to a significant decrease of *MGAT5* mRNA expression (Supplementary Figure 4C), and no significant differences in β1,6 branched N-glycans on E-cadherin were verified (Figure 5C).

To further clarify this potential interplay between branched N-glycans and O-mannosyl glycans expression on E-cadherin, another cell line model was necessary. The MKN45 gastric cancer cell line stably transfected with MGAT5 and thus with overexpression of GnT-V-mediated β1,6 GlcNAc-branched N-glycans was used. Interestingly, Figure 5D demonstrated that the overexpression of *MGAT5* leads to a significant increased modification of E-cadherin with GnT-V-mediated branched N-glycans concomitantly with a significant decrease of O-mannosyl glycans expression on E-cadherin. No significant differences were observed on POMT2 mRNA expression after MGAT5 overexpression (Supplementary Figure 4D).

Taken together, these results suggest the existence of an inverse relationship between E-cadherin N-glycosylation mediated by GnT-V and O-mannosylation that appears to occur at the E-cadherin post-translational level and not necessarily at the genetic level.

Recently, we demonstrated that among the four potential N-glycosylation sites of E-cadherin, Asn-554 is the selected site modified with β1,6 GlcNAc-branched N-glycans resulting in deleterious effects on E-cadherin functions in cancer (17). Moreover, we also

reported that preventing this aberrant glycosylation mediated by GnT-V at Asn-554 improves E-cadherin biological functions in cancer (17). Taking into consideration these results, and in order to evaluate the relationship between E-cadherin O-mannosylation and E-cadherin N-glycosylation at a site-specific level, we analyzed the expression of O-mannosyl glycans in different E-cadherin N-glycan mutant forms. The results showed that E-cadherin M1 mutant, with mutation on Asn554 and displaying no reactivity to L-PHA lectin (no expression of branched N-glycans) exhibited an increased presence of O-mannosyl glycans on E-cadherin as detected by the higher reactivity to Con A lectin (after removal of N-glycans) when compared to E-cadherin WT and M234 (Figure 5E). In contrast, E-cadherin M234 mutant, displaying Asn554 occupancy with branched N-glycans catalyzed by GnT-V exhibited the lowest expression levels of O-mannosyl glycans on E-cadherin. These results strongly support that deletion of Asn-554, which precludes the addition of β1,6 GlcNAc branched N-glycans at this specific site, induces an increased modification of E-cadherin with O-mannosylation. And, the presence of β1,6 GlcNAc branched N-glycans at E-cadherin Asn-554, are remarkably associated with a decreased O-mannosylation on E-cadherin in cancer. Additionally, our results further showed that removal of N-glycans by PNGase F treatment lead to a lower mobility shift of E-cadherin WT and M1 band comparing with E-cadherin M234, indicating that these E-cadherin N-glycoforms WT and M1 may undergo further modification that E-cadherin M234 does not (Figure 5E).

Inverse relationship between branched N-glycans and O-mannosyl glycans in the stomach of transgenic mice models and in human gastric cancer.

In order to assess *in vivo* the biological relevance of afore described crosstalk between these two major types of glycosylation of E-cadherin, we used both *MGAT5* transgenic mice models and human gastric carcinomas clinical samples.

Previously, we have demonstrated that GnT-V-mediated glycosylation causes an abnormal pattern of E-cadherin expression in the gastric mucosa of GnT-V transgenic mice (17). In this study, we evaluated the correlation between the expression of GnT-V-mediated glycosylation and O-mannosylation in the gastric mucosa of *MGAT5* knockout (KO) mice and *MGAT5* overexpressing mice comparing with wild-type (WT) (Figure 6A). The results showed that gastric mucosa of *MGAT5* KO, characterized by no reactivity to L-PHA lectin (no synthesis of β1,6 GlcNAc branched N-glycans) and displaying a normal E-cadherin expression in the basolateral cell membrane, showed higher levels of O-mannosylated proteins, detected by immunoreactivity to T[α1-Man] antibody. On the other hand, mice overexpressing *MGAT5* with a strong staining to L-PHA lectin and an aberrant pattern of E-

cadherin expression, displayed reduced levels of O-mannosylated proteins. These results indicated that MGAT5 expression affects the expression of O-mannosylated proteins.

In a human context, we have previously reported that GnT-V-mediated branched glycosylation is a fundamental mechanism of E-cadherin dysregulation in gastric carcinogenesis, associated with a poorer survival rate of patients. (14, 17). In this study, we evaluated whether the O-mannosylation profile is affected in these gastric cancer patients. The results presented on Figure 6B, showed that human normal gastric mucosa displayed almost no reactivity to L-PHA lectin, but a high positivity to T[α-Man] antibody, indicating high expression of O-mannosylated proteins concomitantly with negligible levels of proteins modified with β1,6GlcNAc-branched N-glycans. In a cancer context, the opposite was observed. Neoplastic cells of diffuse gastric carcinoma with an abnormal/aberrant pattern of E-cadherin expression, showed high levels of expression of β1,6GlcNAc-branched N-glycans and an insignificant expression of O-mannosylated proteins (negative T[α-Man] reactivity). Furthermore, cells from normal adjacent mucosa with a normal cell membrane localization of E-cadherin exhibited no reactivity to L-PHA together with positivity to T[α-Man] antibody, indicating the expression of O-mannosylated proteins but not β1,6GlcNAc-branched N-glycans. Additionally, the L-PHA lectin blotting represented in Figure 6B1 demonstrated high expression levels of overall β1,6GlcNAc-branched N-glycans on diffuse gastric cancer lysates, when compared with normal gastric mucosa, further corroborating the existence of an inverse correlation between O-mannosyl glycans (Figure 1C) and branched N-glycans (Figure 6B1) in diffuse gastric carcinomas. .

Overall, these results support the existence of biological interplay between GnT-V-mediated glycosylation and O-mannosylation affecting the critical functions of a key tumor suppressor protein in gastric carcinogenesis, E-cadherin.

Discussion

E-cadherin dysregulation is a hallmark of cancer development and progression and glycosylation modifications have been recently pinpointed as a fundamental mechanism of E-cadherin regulation with promising clinical applications (11).

The molecular mechanisms that govern E-cadherin regulation in cancer through the newly described O-mannosyl glycans is far from being elucidated (19). Moreover, the potential interplay between the two major types of E-cadherin post-translational modifications, N-glycosylation and O-mannosylation, and its relevance in cancer is completely unknown.

In this study we have demonstrated that the pattern of O-mannosylation in gastric cancer is overall reduced, both at the glycan level as well as at the protein (POMT2) and genetic

level (POMT2 gene). Particularly, we found that E-cadherin suffered a decreased O-mannosylation that accompanied the acquisition of a poorly-differentiated cancer phenotype. This decrease in the O-mannosyl glycans attached to E-cadherin resulted in the impairment of E-cadherin functions in cancer cells through interfering in its normal cellular localization and in the stability of adherens junctions. Therefore, a novel functional link is established between E-cadherin dysfunction in cancer and reduced O-linked mannose residues attached to the glycoprotein. When O-mannosylation was promoted within an undifferentiated gastric cancer phenotype, a recovery of E-cadherin membrane localization and biological functions was observed. The increased modification of E-cadherin with O-mannosyl glycans promotes a correct localization of E-cadherin at the cell-cell contacts together with an improvement in the molecular assembly and stability of the intercellular adhesive complex. These results are in accordance with Lommel et al. that described that O-mannosylation of E-cadherin is highly relevant to the formation and maintenance of adherens junctions during the morula-to-blastocyst transition and in normal MDCK cells (18).

In addition, we here provide new evidences for the existence of a yet uncovered interplay between O-mannosylation and N-glycosylation that appears to operate in a coordinated and site-specific manner on E-cadherin glycoprotein, with impact in the modulation of its critical functions in gastric cancer.

In fact, GnT-V-mediated branched N-glycosylation has been described as an instrumental mechanism of E-cadherin dysregulation in cancer (11, 12, 34, 35). The modification of E-cadherin with β1,6 GlcNAc branched N-glycans was observed in gastric cancer patients associated with poor survival rates (17) and has been described to induce E-cadherin cellular mislocalization and an incorrect assembly of adherens-junctions, thus compromising cell-cell adhesion and downstream signalling pathways that ultimately contribute to tumour invasiveness and metastases (14, 15, 17, 36).

In the present study, we consistently demonstrated that the manipulation of the O-mannosylation pathway (either by POMT2 silencing or overexpression) in different gastric cancer cellular phenotypes consistently resulted in alterations of the expression of branched N-glycans on E-cadherin in an inverse and coordinated manner. POMT2 knockdown resulted in an increased GnT-V-mediated branched N-glycans on E-cadherin, Likewise, when the branched N-glycosylation pathway is manipulated, by inducing MGAT5 overexpression, a significantly decreased levels of O-mannosyl glycans attached to E-cadherin was observed. This mutual regulatory mechanism between GnT-V-mediated N-glycosylation and O-mannosylation is a new mechanism of E-cadherin functional regulation in cancer. Additionally, we demonstrated that this inverse relationship between the two forms of E-cadherin glycosylation may occur in a site-specific manner.

In a previous work we reported that among the four potential N-glycosylation sites of E-cadherin, the Asn-554 is selectively occupied with β1,6 GlcNAc-branched N-glycans. Preventing the addition of these branched N-glycans at Asn 554 resulted in a protective effect on E-cadherin, precluding its functional dysregulation in cancer (17). In this study, we demonstrated that the presence of the deleterious branched N-glycans at Asn-554 of E-cadherin directly affects its O-mannosylation pattern that becomes reduced. The prevention of this site-specific glycosylation modificationby a mutation (17), potentiates the O-mannosylation profile of E-cadherin associated with a recover of its functions. The presence of β1,6 GlcNAc branched N-glycans at E-cadherin Asn-554 may introduce conformational changes than can induced reduced levels of O-mannosylation.

Interestingly, this coordinated mechanism was further observed *in vivo*, in the stomach of two different MGAT5 transgenic mouse models. The MGAT5 knockout mice characterized by no GnT-V-mediated branched glycosylation, revealed a remarkable reactivity to O-mannosylated proteins. Conversely, MGAT5 overexpressing and WT mice with high to moderate levels of branched N-glycans showed reduced levels of O-mannosylated glycoproteins. Moreover, and relevant from the clinical point of view, this interplay between the two glycosylation forms was also demonstrated in patients with diffuse gastric carcinoma. Gastric neoplastic cells, characterized by an aberrant E-cadherin expression at the cytoplasm (meaning dysfunctional), exhibited increased expression of β1,6 GlcNAc-branched N-glycans concomitantly with negligible levels of O-mannosyl glycans. The opposite was observed in normal gastric mucosa. These results strongly suggest that GnT-V-mediated glycosylation negatively affects protein O-mannosylation and vice-versa.

Taken together, our observations support a new mechanism of E-cadherin regulation in cell biology. In a normal epithelial cell phenotype, E-cadherin undergoes a preferential O-mannosylation modification, which contributes to the proper adhesive functions of E-cadherin that are needed in homeostasis. In the ER lumen, POMT2/POMT1 complex catalyses the addition of mannose residues at the E-cadherin EC4 domain and along the Golgi apparatus E-cadherin does not undergo complex branched N-glycosylation catalysed by GnT-V. These predominance of O-mannosyl glycans rather than complex branched N-glycans contribute to a protective effect on E-cadherin, precluding its functional dysregulation (Figure 7). During malignant transformation, these balanced levels of O-mannosyl glycans and complex branched N-glycans in E-cadherin are lost. In a cancer cell, both the POMT2 gene and enzyme are downregulated and consequently the pattern of E-cadherin O-mannosylation decreases. Further, and owing to increased MGAT5 expression and GnT-V activity frequently observed in a cancer cell, E-cadherin is predominantly targeted by GnT-V-mediated glycosylation at the Golgi apparatus, where β1,6 GlcNAc-

branched N-glycans are added to Asn-554 of E-cadherin. The presence of these deleterious branched N-glycans structures together with reduced levels of O-mannosyl glycans on EC4 ectodomain lead to loss of E-cadherin suppressive functions in cancer, thus contributing to tumour progression and metastases (Figure 7).

Overall, this study proposes a newly identified mechanism of E-cadherin (dys) regulation in cancer that is precisely coordinated through the interplay between O-mannosylation and N-glycosylation machinery that operates at the protein site-specific level and with direct implications in cancer cell biology. Moreover, these differential patterns of E-cadherin glycosylation can constitute promising (glyco) biomarkers for gastric cancer patients´ clinical management.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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Figure Legends

Figure 1

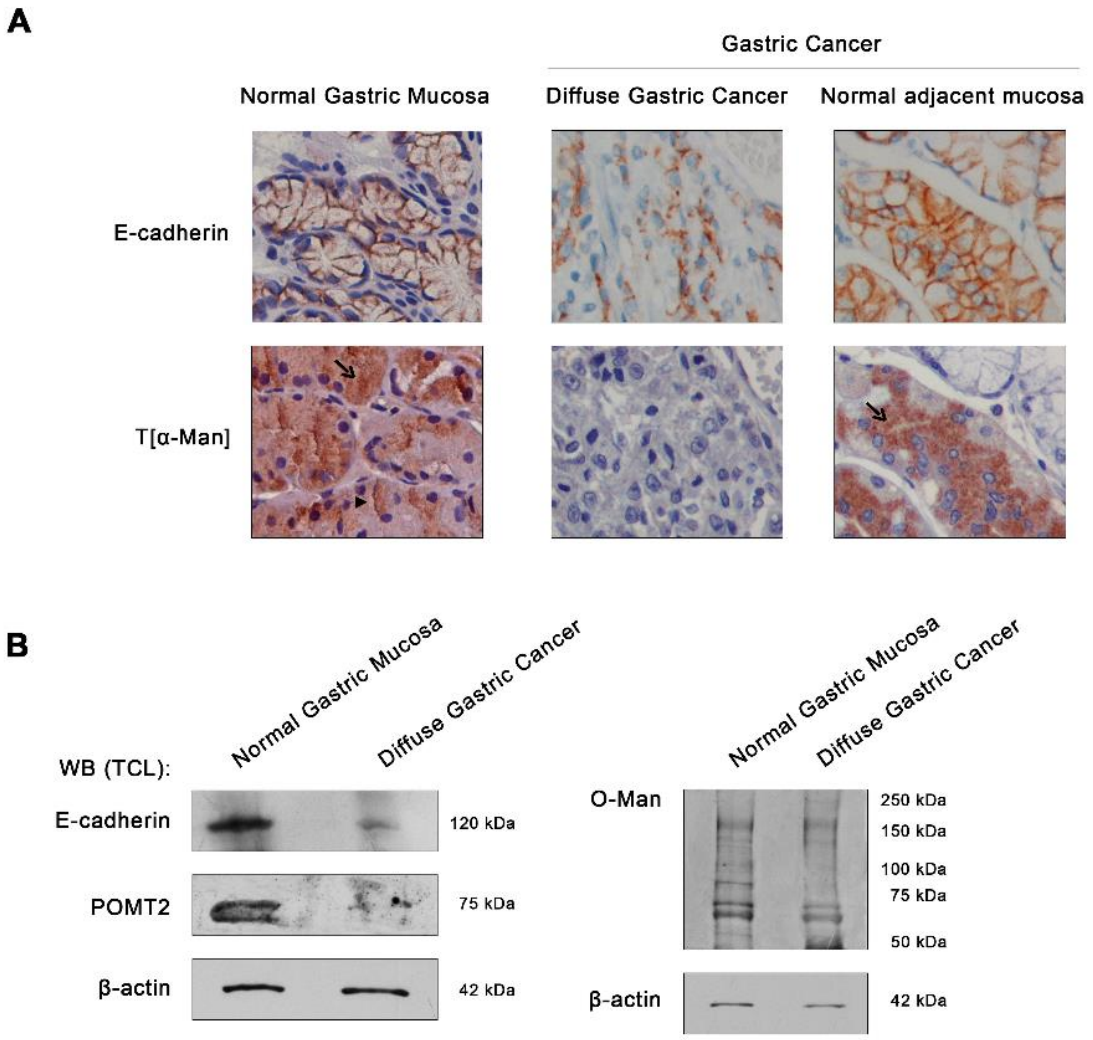

Figure 1. Expression of E-cadherin and O-mannosylated proteins in human normal gastric mucosa and diffuse gastric cancer.

(A) Immunoexpression of E-cadherin and O-mannosylated proteins. Immunohistochemical analysis of the normal gastric mucosa showed E-cadherin expression at the basolateral cell surface, as expected. T[α1-Man] immunohistochemistry detecting O-mannosylated proteins revealed a remarkable staining of O-mannosylated proteins in the cytoplasm (arrow) and in the cell membrane (arrowhead) in the body region of normal gastric mucosa. No reactivity to T[α1-Man] antibody was verified in neoplastic cells displaying an aberrant expression of E-cadherin at the cytoplasm. However, intact glands of normal adjacent mucosa with E-cadherin expression at the cell membrane

exhibited cytoplasmic staining of O-mannosylated proteins, although less intense than normal gastric body mucosa. Original magnification: 600x. (B) Evaluation of POMT2 protein and O-mannosyl glycans expression in total lysate from normal gastric mucosa and diffuse gastric carcinoma tissues. A significant decreased expression of E-cadherin and POMT2 at the protein level was observed in diffuse gastric carcinoma. Then, to evaluate the expression of O-mannosyl glycans, a blotting using Con A mannose-binding lectin was performed. Con A lectin recognizes α-linked mannose residues from O-Man and N-glycoproteins. So, tissue lysates were treated with PNGase F to remove N-glycans before the lectin blotting. As result, lower levels of O-mannosyl glycans expression were detected in diffuse gastric carcinoma, when compared with lysate from normal gastric mucosa. See also Supplementary Figure S1.

Figure 2

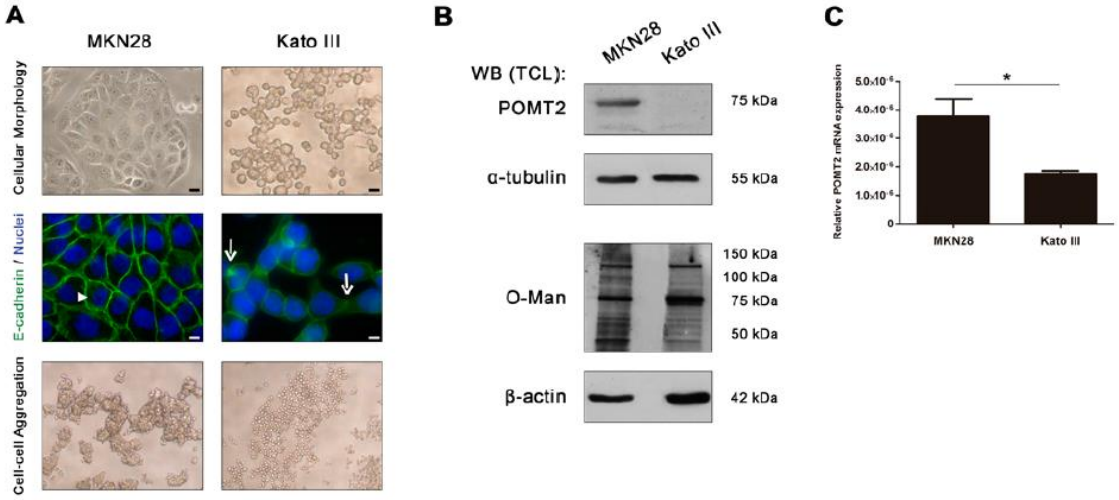

Figure 2. E-cadherin O-mannosylation profile decreases in poorly differentiated gastric cancercells.

(A) Morphology, E-cadherin localization and cell aggregation capacity of MKN28 and Kato III cell lines. MKN28 cells are characterized by an epithelial morphology and the capacity to form large compact cell aggregates. E-cadherin immunofluorescence revealed a strong membranous staining in MKN28 cells (arrowhead). In contrast, Kato III cell line displayed a spherical morphology and a decreased cell aggregation capacity, when compared with MKN28 cells. Regarding the E-cadherin expression, Kato III cells exhibited an abnormal localization of E-cadherin at the cell-cell contacts with some cytoplasmic staining (arrow). White size bars ~ 5μm. (B) Evaluation of POMT2 protein and O-mannosyl glycans expression in the total cell lysates from MKN28 and Kato III cells. A significant

decrease of POMT2 protein and O-mannosyl glycans expression were observed in total cell lysate from Kato III, when compared with MKN28. (C) POMT2 mRNA expression. POMT2 mRNA expression was significantly decreased in Kato III cells (P= 0,0293). The data presented is referred to three independent biological replicates. (D) E-cadherin O-mannosylation decreases in poorly differentiated gastric cancer cell line. E-cadherin immunoprecipitated from MKN28 cells showed an increased Con A lectin reactivity, after *PNGAse F* digestion, when compared with E-cadherin immunoprecipitated from Kato III cells, revealing a significant increase in O-mannosyl structures modifications (4,4-fold; *P*= 0,0081). Bar graphs, amounts of O-mannosyl glycans on E-cadherin were determined from the ratios of densities of Con A lectin reactivity (with PNGase F treatment) after normalization to E-cadherin. Results are described as mean ± s.d of two independent experiments. See also Supplementary Figure S2.

Figure 3

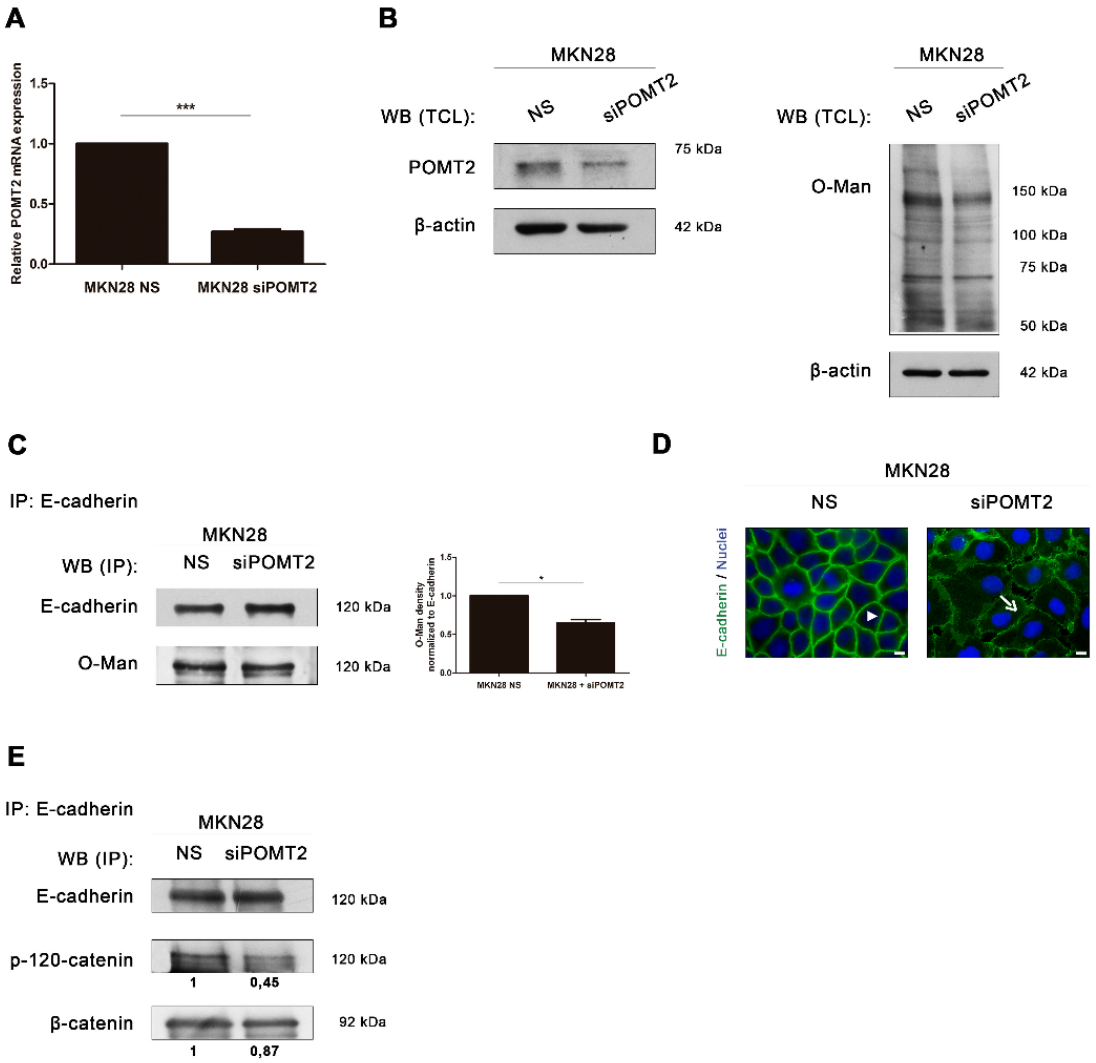

Figure 3. POMT2 knockdown impairs E-cadherin normal localization and assembly of the E-cadherin-catenin complex by reducing O-mannosyl glycans on E-cadherin

(A) *POMT2* mRNA expression of MKN28 cells after POMT2 silencing by using the small interfering RNA (siRNA) technique. Around 75% of POMT2 silencing was observed. NS, non-silencing. The relative POMT2 mRNA expression of siPOMT2 cells is expressed as the fold increase, compared with non-silencing cells, which was taken as 1. The data presented is referred to three independent biological replicates. (B) Immuno- and lectin blot analysis of POMT2 protein and O-mannosyl glycans on total cell lysate from MKN28 cells after *POMT2* silencing. A significant decreased of POMT2 protein and O-mannosyl glycans expression after *POMT2* silencing were observed. (C) *POMT2* silencing resulted in a significant decreased Con A-mannose binding lectin reactivity for the E-cadherin band after removal of N-glycans. Bar graphs, amounts of O-mannosyl glycans on E-cadherin were

determined from the ratios of densities of lectin reactivity (with PNGase F treatment) after normalization to E-cadherin. Results are described as mean ± s.d of two independent experiments. *P*-value= 0,05. (D) Immunofluorescence analysis of MKN28 cells after *POMT2* silencing demonstrated a reduced and incomplete E-cadherin localization at the cell membrane. White size bars ~ 5µm. (E) E-cadherin immunoprecipitation followed by p-120-catenin and β-catenin western-blot after *POMT2* silencing. Knockdown of *POMT2* resulted in a decreased interaction between E-cadherin and p-120-catenin (of about 0,55-fold). No significant differences were observed regarding the interaction of E-cadherin with β-catenin after *POMT2* knockdown. Amount of association were determined from the ratios of densities of p-120-catenin and β-catenin after normalization to E-cadherin.

Figure 4

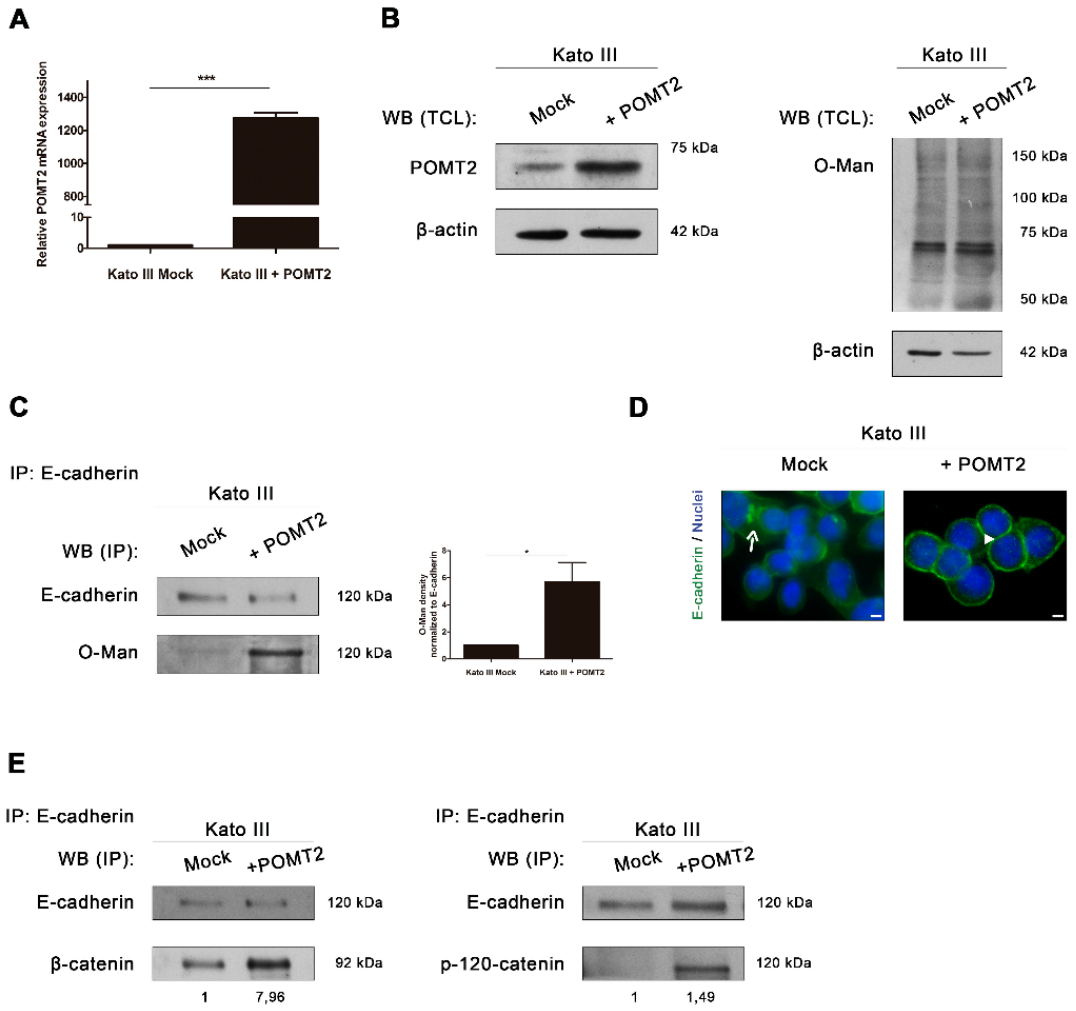

Figure 4. POMT2 overexpression induces the recovery of E-cadherin biological functions.

(A) Confirmation of the overexpressed *POMT2* mRNA transcripts in Kato III cells. The relative POMT2 mRNA expression is expressed as the fold increase, compared with mock cells, which was taken as 1. The data presented is referred to three independent biological replicates. (B) Overexpression of *POMT2* induces an increased expression of POMT2 at the protein level and O-mannosyl glycans. (C) Evaluation of the impact of *POMT2* overexpression in E-cadherin O-mannosylation. *POMT2* overexpression leads to a significant increased reactivity of E-cadherin immunoprecipitated to Con A lectin blotting after PNGase F treatment, suggesting an increase of E-cadherin O-mannosylation (around 5.7- fold). Bar graphs, amounts of O-mannosyl glycans on E-cadherin were determined from the ratios of densities of lectin reactivity (with PNGase F treatment) after normalization to E-cadherin. Results are described as mean ± s.d of two independent experiments. *P*-value=

0,0376 (D) Immunofluorescence analysis of Kato III cells overexpressing *POMT2* demonstrated an increased localization of E-cadherin at the cell-cell membrane. White size bars ~ 5µm. (E) Evaluation of β-catenin and p-120-catenin recruitment by E-cadherin after *POMT2* overexpression. Kato III cells overexpressing POMT2 displayed an increased interaction between E-cadherin and β-catenin and p-120-catenin (around 8.0-fold and 1.5-fold, respectively). See also Supplementary Figure S3.

Figure 5

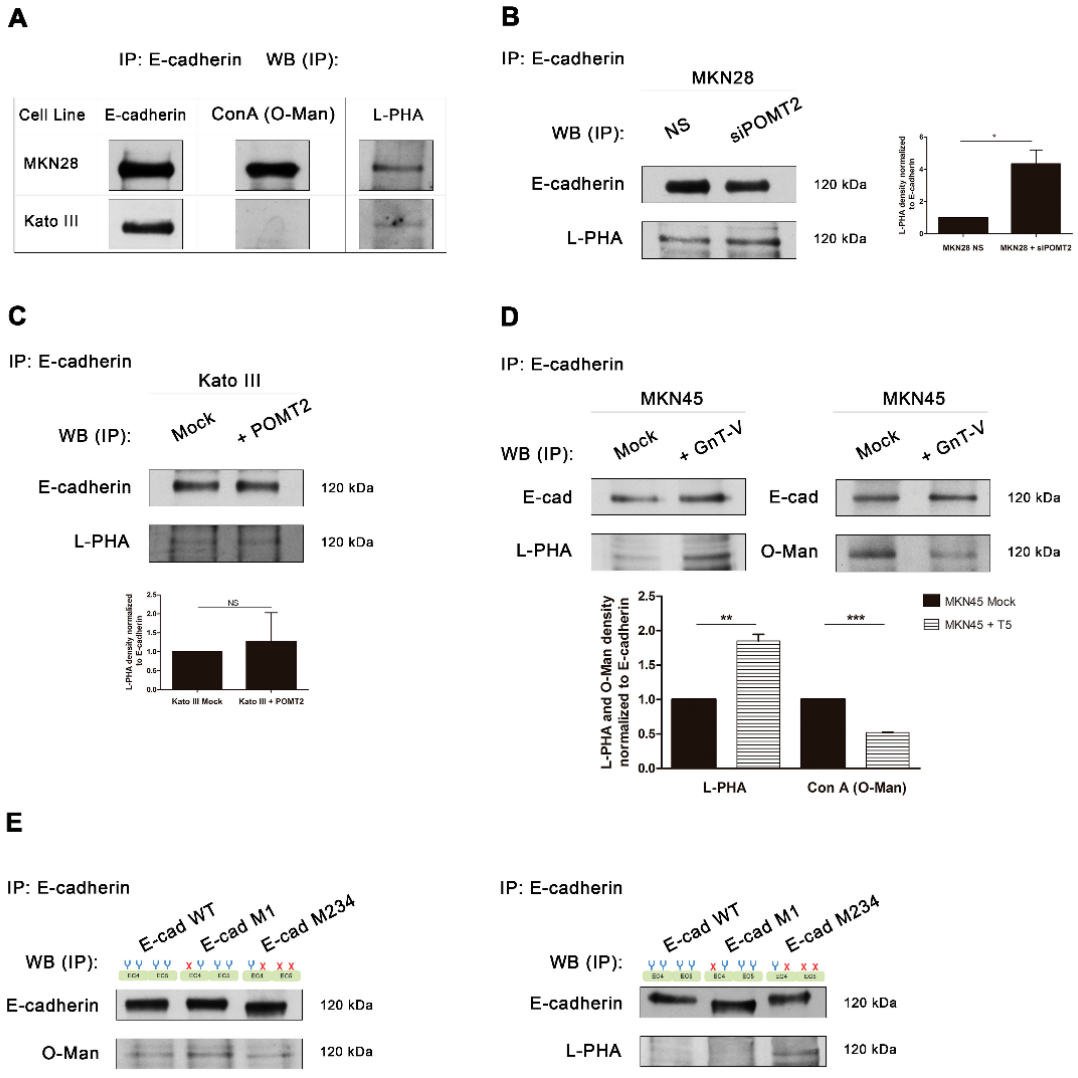

Figure 5. Interplay between GnT-V-mediated E-cadherin N-glycoylation and POMT2-mediated E-cadherin O-mannosylation in vitro.

(A) Evaluation of reactivity of E-cadherin immunoprecipitated from MKN28 and Kato III cells to Con A (after removal of N-glycans) and L-PHA lectins. E-cadherin

immunoprecipitaded from MKN28 cells and Kato III cells displayed a higher reactivity to Con A and L-PHA lectins, respectively. (B and C) Reactivity of E-cadherin immunoprecipitated after POMT2 knockdown (B) or POMT2 overexpression (C) to L-PHA lectin. Knockdown of POMT2 resulted in an increased expression of β1,6 GlcNAc branched N-glycans on E-cadherin (around 4.0-fold; *P*-value=0,0250. Bar graphs, amounts of β1,6 GlcNAc branched N-glycans on E-cadherin were determined from the ratios of densities of L-PHA lectin reactivity after normalization to E-cadherin. Results are described as mean ± s.d of two independent experiments. (D) Overexpression of MGAT5 in MKN45 cells induced an increased positivity to L-PHA lectin (1,85-fold) and a decreased reactivity to Con A lectin after PNGase F treatment (0,52-fold). Bar graphs, amounts of β1,6 GlcNAc branched N-glycans and O-mannosyl glycans on E-cadherin were determined from the ratios of densities of Con A and L-PHA lectins reactivity (with PNGase F treatment) after normalization to E-cadherin. Results are described as mean ± s.d of two independent experiments. (E) Evaluation of E-cadherin O-mannosylation and E-cadherin N-glycosylation mediated by GnT-V regarding site-specific N-glycosylation occupancy. E-cadherin M1 immunoprecipitated exhibited an increased reactivity to Con A-mannose binding lectin (after PNGase F treatment) when compared with E-cadherin WT and M234. See also Supplementary Figure 4. Lectin histochemistry detecting β1,6 GlcNAc-branched N-glycans demonstrated higher reactivity of E-cadherin M234 to L-PHA lectin. In addition, E-cadherin M1 showed an increased mobility shift compared with E-cadherin WT and M234.

Figure 6

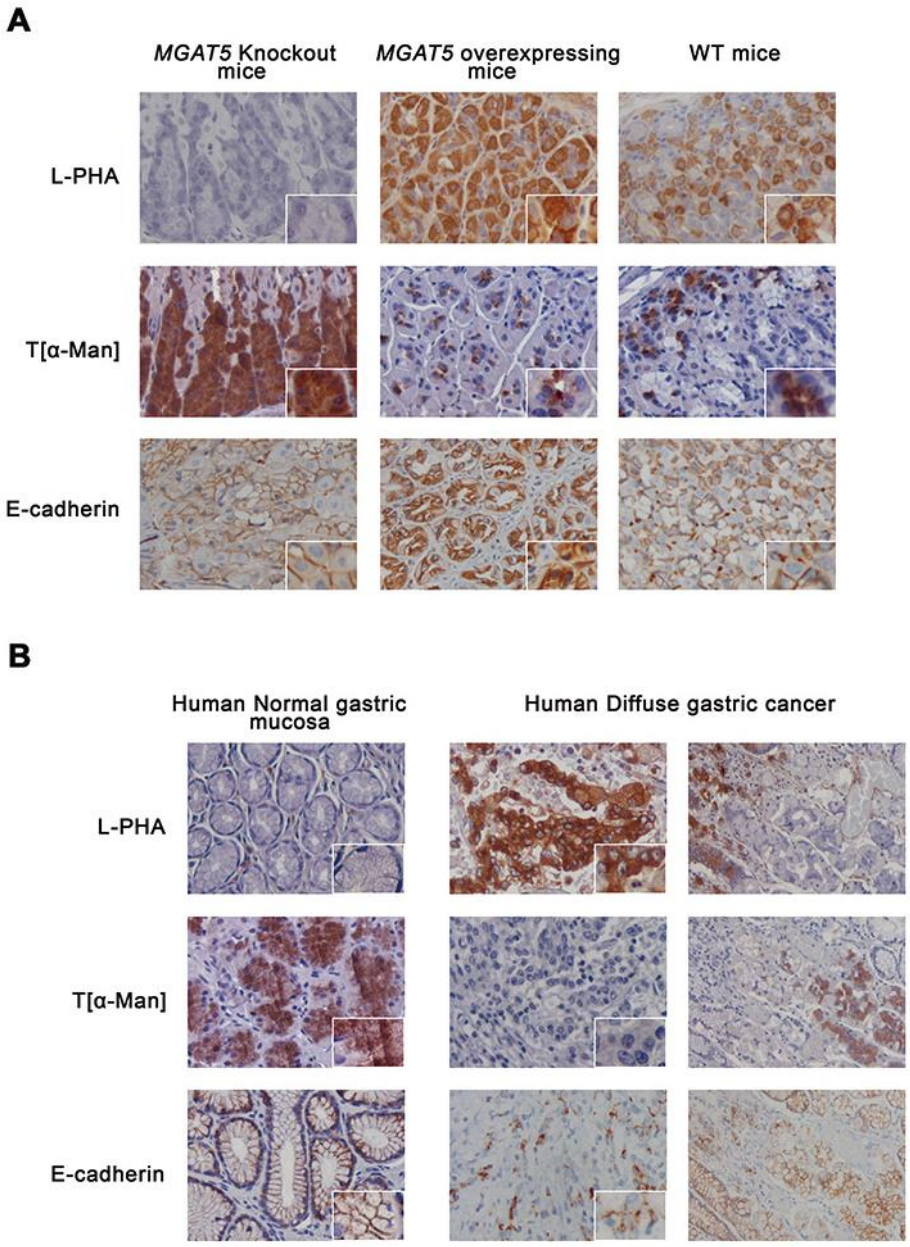
A
MGAT5 Knockout mice
MGAT5 overexpressing mice
WT mice
L-PHA
T[α-Man]
E-cadherin
B
Human Normal gastric mucosa
Human Diffuse gastric cancer
L-PHA
T[α-Man]
E-cadherin
B1
WB (TCL):
Normal gastric mucosa
Diffuse gastric cancer
L-PHA
150 kDa
100 kDa
75 kDa
50 kDa
α-tubulin
55 kDa

Figure 6. The interplay between GnT-V – mediated N-glycosylation and O-mannosylation in vivo.

Evaluation of the expression of β1,6 GlcNAc-branched structures and O-mannosylated proteins in the gastric mucosa of *MGAT5* knockout (KO) and *MGAT5* overexpressing mice and in human normal gastric mucosa versus gastric carcinoma. (A) Histochemical analysis detecting β1,6 GlcNAc-branched N-glycans showed an absent, a strong, and a moderate L-PHA lectin staining in the gastric mucosa of *MGAT5* KO, *MGAT5* transgenic, and WT mice, respectively. Concerning the T[α-Man] immunoreactivity, gastric mucosa from WT and *MGAT5* transgenic mice showed low expression of O-mannosylated proteins. In contrast, MGAT5 KO gastric mucosa displayed a clear overexpression of O-mannosylated proteins. E-cadherin is expressed at the cell membrane of WT and MGAT5 KO, while in *MGAT5* transgenic mice E-cadherin is also aberrantly expressed at the cytoplasm. (B) Evaluation of the interplay between N-glycosylation and protein O-mannosylation in human gastric mucosa. L-PHA histochemistry detecting the β1,6 GlcNAc-branched N-glycans catalyzed by GnT-V showed no reactivity in the human normal gastric mucosa and a marked positivity in neoplastic cells from gastric carcinoma. Regarding the expression of O-mannosylated proteins, we verified that neoplastic cells from diffuse gastric carcinoma positive to L-PHA lectin and showing an aberrant expression of E-cadherin at the cytoplasm, exhibited no reactivity to T[α-Man] antibody. In contrast, cells from normal adjacent mucosa and human normal gastric mucosa characterized by a cell membrane localization of E-cadherin and almost no expression of β1,6 GlcNAc-branched N-glycans, are positive to T[α-Man] antibody. Original magnification: 400x (B1) Evaluation of β1,6 GlcNAc-branched glycans expression in total lysate from normal gastric mucosa and diffuse gastric carcinoma tissues. An increased expression of β1,6 GlcNAc-branched N-glycans detected by L-PHA lectin blotting was verified in lysate from diffuse gastric carcinoma.

Figure 7

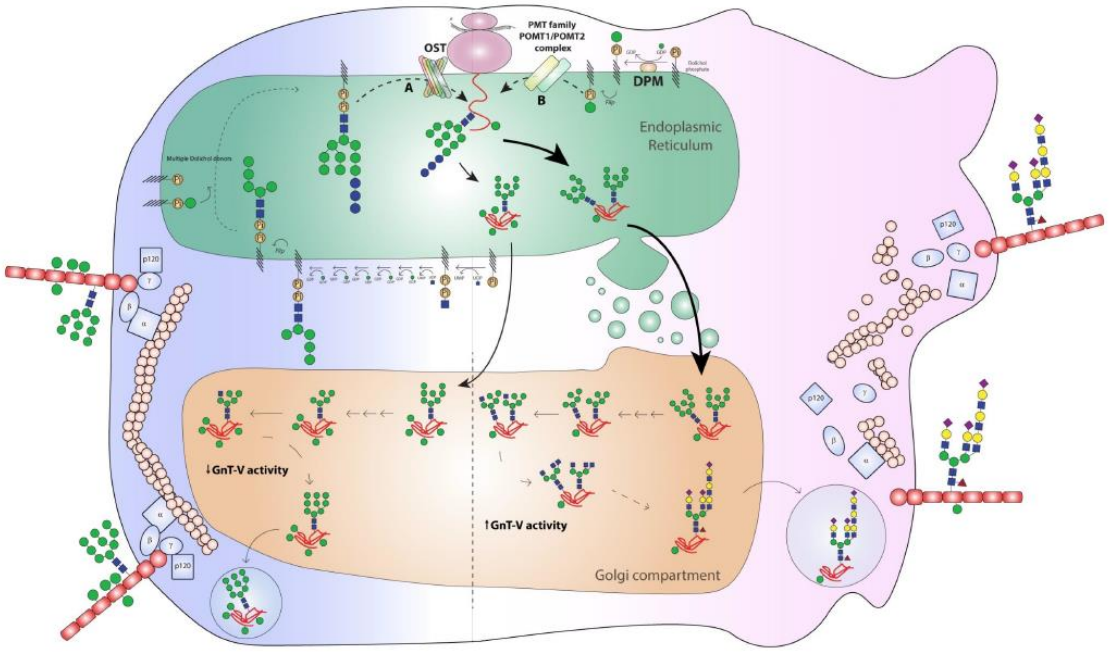

Figure 7. Proposing model for the pattern of E-cadherin O-mannosylation and the interplay with GnT-V-mediated N-glycosylation in a cancer context.

In a normal epithelial cell, E-cadherin undergoes a preferential O-mannosylation modification contributing to the proper adhesive functions of E-cadherin. In the ER lumen, the mannose residues are added at the E-cadherin EC4 domain by action of POMT2/POMT1 complex, and along the Golgi apparatus E-cadherin does not undergo complex branched N-glycosylation catalysed by GnT-V. The predominance of O-mannosyl glycans rather than complex branched N-glycans contributes to a protective effect on E-cadherin. During malignant transformation, both the POMT2 gene and enzyme are downregulated and consequently the pattern of E-cadherin O-mannosylation decreases. Further, and owing to increased MGAT5 expression and GnT-V activity frequently observed in a cancer cell, E-cadherin is predominantly modified with β1,6 GlcNAc-branched N-glycans at E-cadherin Asn-554 by action of GnT-V. The presence of these deleterious branched N-glycans structures combined with reduced levels of O-mannosyl glycans on EC4 ectodomain lead to loss of E-cadherin suppressive functions in cancer, thus contributing to tumour progression and metastases.

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Chapter V

General Discussion

Cancer is an important health problem that causes substantial patient death worldwide due to its incidence, prevalence and survival. Regarding gastric cancer, it is considered the sixth most common type of cancer, and is the fourth leading cause of cancer-related worldwide [1]. Despite declining trend in incidence of gastric cancer, the outcomes of patients remain poor due to lack of effective biomarkers to early detect gastric cancer [2, 3]. In recent years, with the development of modern biomedical technologies, several studies provided new insights about novel biomarkers with diagnostic and prognostic value, such as cancer antigen 19-9 (CA19-9) – Sle_a antigen, a serological marker used in gastric cancer [4-6]. However, owing to the low sensitivity and specificity required for early detection of gastric carcinoma, the discovery of new cancer specific biomarkers are needed in order to improve early gastric cancer diagnosis, prognosis, and the development of new and targeted-specific therapies.

Advances about molecular events of gastric cancer, involving the alteration of pathogenesis, invasion and metastasis, have been enabling continuous efforts to identify molecular biomarkers for early gastric cancer diagnosis [3]. Regarding the diffuse subtype of gastric cancer, the main molecular carcinogenic event is the reduction of intercellular adhesiveness in the non-cohesive neoplastic cells [7]. Defective intercellular structures and cell-cell adhesion can lead to the dissociation of the primary tumour, and to the ability of neoplastic cells to invade surrounding tissues and to metastasize. These neoplastic properties have been reported to be closely related with dysregulation of the invasion suppressor protein- E-cadherin [8, 9].

E-cadherin, a calcium-dependent cell-cell adhesion molecule, plays major roles in maintaining the epithelial tissue architecture and cell polarity [10, 11]. The downregulation or inactivation of E-cadherin functions in diffuse gastric cancer have been reported to occur through various genetic/epigenetic mechanisms such as: somatic mutations of the *CDH1* gene [12], *CDH1* transcriptional silencing [13], miRNAs [14], *CDH1* promoter hypermethylation [15, 16], and E-cadherin trafficking dysregulation [17, 18]. Besides these genetics and epigenetics mechanisms, E-cadherin-mediated carcinogenesis has been also associated with aberrant activation of signalling pathways and interaction with cytoskeleton components, integrin, growth-factors receptors, among others [19-22] Nevertheless, there is still a high percentage of human sporadic gastric cancer cases (around 70%), predominantly diffuse subtype, which displayed E-cadherin dysfunction that is not explained

by any of the aforementioned mechanisms of E-cadherin epi/genetic dysregulation [23]. This clinical gap of knowledge fosters the identification of an alternative mechanism responsible for the dysregulation of E-cadherin in a gastric cancer setting. We and others have been proposed an alternative post translational mechanism of E-cadherin dysregulation in cancer- the glycosylation [24]. Glycans are involved in fundamental molecular and cell biology processes occurring in cancer, such as cell-adhesion [25-27], cell-matrix and host-pathogen interactions [25], immune modulation [28, 29], endocytosis, and signal transduction [30]. Therefore, minor alterations in cell glycan signature may support neoplastic progression by promoting proliferation, invasion, metastasis, and angiogenesis [30, 31].

E-cadherin is a glycoprotein that is post-translationally modified by glycosylation. During malignant transformation, E-cadherin undergoes an extensive modification on its N-glycosylation profile which regulates the tumour cell-cell adhesion by interfering with its biological functions [32]. Moreover, the adhesive function of E-cadherin has been described to be influenced by the pattern of N-glycosylation through modifying the assembly and stability of the adherens junctions [33, 34]. Liwosz et al. reported that modification of E-cadherin with complex N-glycans are associated with formation of dynamic but immature adherens junctions whereas reduced pattern of E-cadherin N-glycosylation promotes the establishment of stable adherens junctions [33]. The extension of N-glycans branching are thus inversely related with the epithelial adhesion [35]. According to the literature, there are two major forms of complex N-glycans that modify E-cadherin protein: the bisecting GlcNAc N-glycans structures, catalysed by GnT-III, and the β1,6 GlcNAc-branched N-glycans, catalysed by GnT-V [26, 27]. Pinho et al reported a regulatory mechanism between E-cadherin expression, and the remodelling of its N-glycan structures through the enzymatic competition between GnT-III and GnT-V in cancer [27, 36].

Taking into account the literature about E-cadherin N-glycosylation mediated by GnT-III, some questions arise and that need to be further elucidated. Which signalling pathway-dependent E-cadherin is involved in the bidirectional crosstalk between E-cadherin and the enzyme GnT-III? Furthermore, concerning the GnT-V-mediated E-cadherin N-glycosylation, predominant in human gastric cancer patients, which is the precise N-glycosylation site of E-cadherin modified with these deleterious N-glycans? Having the new evidences about E-cadherin O-mannosylation in homeostasis, how does O-mannosylation contribute to the E-cadherin regulation in cancer?

Chapter II- Insulin/IGF-I Signalling Pathways Enhances Tumour Cell Invasion through Bisecting GlcNAc N-glycans Modulation. An Interplay with E-Cadherin

E-cadherin and GnT-III expression are involved in a bidirectional cross-talk where E-cadherin mediated cell-cell adhesion regulates *MGAT3* transcription [27], increasing GnT-III expression and its products (bisecting GlcNAc N-glycans) on E-cadherin [27, 37, 38], which in turn downregulates the tyrosine phosphorylation of β-catenin, contributing to suppression of tumour invasion and metastasis [39]. However, it remained to identify the signalling pathway (E-cadherin dependent) involved in this functional crosstalk. Therefore, the chapter II of this PhD thesis aimed to assess the underlying signalling pathway involved in the regulation of the bisecting GlcNAC N-glycans in the process of tumour progression of epithelial cells.

Phosphoproteome analysis showed that MDA-MB-435 cancer cells, that endogenously lacks E-cadherin expression, displayed a significant increased phosphorylation of insulin receptor (IR)/ IGF-I receptor (IGF-IR) receptors tyrosine kinase (RTK). Moreover, after exogenous E-cadherin expression, IR/IGF-IR phosphorylation levels are remarkably inhibited. These results suggested an inhibitory effect of E-cadherin in the activity of Insulin and IGF-I signalling pathways. According to the literature, Insulin/IGF-I signalling pathway has been strongly associated with malignant transformation [40], being also related with loss of epithelial features of carcinoma cells [41, 42]. IGF-I was reported to increase the invasive potential inducing TGF-β1-mediated EMT and decreased E-cadherin expression [41]. Besides modulation of Insulin/IGF-I signalling pathway, decreased phosphorylation levels of ERK ½ were also verified upon E-cadherin overexpression. So, E-cadherin expression in MDA-MD-435 cancer cells induces a downregulation of Ras/Raf/MEK/ERK downstream signalling pathway through the significant decreased activity of the IR/IGF-IR signalling.

More importantly, we further demonstrated that the IR/IGF-IR signalling pathway also plays a role in the modulation of the GnT-III-mediated N-glycosylation in general and specifically on E-cadherin. Stimulation of IR/IGF-IR signalling induced a decreased overall expression of bisecting GlcNAc N-glycans and specifically on E-cadherin glycoprotein, promoting a mislocalization of E-cadherin into the cytoplasm. These results suggest that the bidirectional crosstalk between E-cadherin and bisecting GlcNAc N-glycans is mediated by the IR/IGF-IR signalling pathway. Furthermore, we observed that IR/IGF-IR activation also modulates the expression of epithelial and mesenchymal markers and induces increased capacity of tumour cell invasion.

In summary, the work presented in chapter II supported a close interplay between E-cadherin, its glycosylation with bisecting GlcNAc N-glycans and IR/IGF-IR signalling pathway in the process of tumour cell invasion, further clarifying the positive feedback loop established between E-cadherin and GnT-III that modulates E-cadherin-mediated cell-cell adhesion. In the absence of IR/IGF-IR signalling, E-cadherin induces the expression of bisecting GlcNAc N-glycans overall and specifically on E-cadherin, contributing to the stabilization of E-cadherin at the cell membrane, and at last promoting tumour suppression. In a cancer context, upregulation of Ras/Raf/MEK/ERK induces IR/IGF-IR signalling activation which in turn decrease the expression of bisecting GlcNAc N-glycans in general and specifically on E-cadherin, leading to E-cadherin mislocalization into the cytoplasm and the expression of mesenchymal markers that favour tumour cell invasion.

The further contribution of GnT-III-mediated N-glycosylation in tumour suppression was also supported with our previous observations showing an inhibition of E-cadherin endocytosis, delayed turnover of E-cadherin at the cell surface, and improvement of intercellular adhesion by recruitment of catenins [26]. Furthermore, *MGAT3* glycogene expression and GnT-III-mediated E-cadherin glycosylation also contribute to an epithelial-like phenotype that prevents the EMT process [43, 44].

Chapter III - Preventing E-cadherin aberrant N-glycosylation at Asn-554 improves its critical function in gastric cancer

The enzymatic activity of GnT-V is known to be upregulated in gastric cancer [45], contributing to cancer cell invasion and metastasis [46]. Pinho et al reported that GnT-V-mediated E-cadherin N-glycosylation interfere with β-catenin and p120-catenin recruitment, disturbing the stability of the adherens junctions and impairing normal E-cadherin cellular localization, ultimately compromising the E-cadherin-mediated cell-cell adhesion [26]. In addition, studies demonstrated that β1,6 GlcNAc branched N-glycans on cadherins reduces the N-cadherin clustering at the cell surface and compromises the outside-in-signal transduction-pathway of ERK-mediated by N-cadherin [47], contributing to increased cell migration and invasion [48]. In a gastric cancer setting in which E-cadherin display aberrant expression not due to genetic and structural alterations, the gastric cancer cells exhibit an increased expression of β1,6 GlcNAc branched N-glycans in general and specifically on E-cadherin [26]. Taken together is of utmost relevance for the clinic the precise identification and characterization of the site-specific E-cadherin N-glycans occupancy that functionally impact the E-cadherin functions. The study presented in chapter III intended to determine

the role that site-specific glycosylation of E-cadherin has in its defective function in a gastric cancer context.

In this study, we demonstrated that human patients with diffuse gastric cancer displayed an aberrant expression of E-cadherin which is specifically modified with β1,6 GlcNAc N-glycans structures that is correlated with a poorer survival rate of patients, when compared with diffuse gastric cancer patients without aberrant modifications mediated by GnT-V on E-cadherin. This specific E-cadherin aberrant glycoform *in vivo* was thus showed to be important in the pathogenesis of gastric carcinoma and in the survival rates of patients.

The human E-cadherin ectodomain comprises four potential N-glycosylation sites: two putative sites at the EC4 subdomain (Asn-554 and Asn-566) and the remaining two potential sites at the EC5 subdomain (Asn-618 and Asn-633) [36]. Previous reports demonstrated that the N-glycan at Asn-633 is required for E-cadherin expression, folding and trafficking [49]. Unglycosylation of E-cadherin at this specific site induces its arrest in ER as a misfolded protein being targeted via ERAD pathway [49]. In turn, N-glycans situated at Asn-554 and at Asn-566 in human breast carcinoma cell line were described to be important for the cell cycle progression [50], and for the adhesive function of E-cadherin [34].

In the study presented on chapter III, an *in silico* bioinformatics analysis combined with the *in vitro* site-directed mutagenesis approach in a gastric cancer cell line revealed that among the four potential N-glycosylation sites available in the extracellular domain of E-cadherin, the sites Asn-554 and Asn-633 are N-glycosylated and the other two potential sites (Asn-566 and Asn-618) are not occupied with N-glycans. Furthermore, the enzymatic digestion approach showed that Asn-554 displayed complex type N-glycans while Asn- 633 is occupied with high mannose N-glycans. Ultimately, the glycomics analysis by advanced mass spectrometry approach corroborated these results revealing that E-cadherin WT and M234 showed higher levels of sialylated and complex-type N-glycans. Our observations combined with the literature indicated that the E-cadherin N-glycan site occupancy is tissue specific.

Importantly, we further identified that Asn-554 is the selected site modified with β1,6 GlcNAc-branched N-glycans, catalysed by GnT-V, being the key site for the functional dysregulation of E-cadherin in gastric tumour cells. This site-specific N-glycosylation mediated by GnT-V compromises the cis-dimerization capability, decreases the rate of cell-cell aggregation and interferes with the molecular assembly and stability of the adhesion complexes. Consistent with our observations, several reports demonstrated that

modification of E-cadherin with β1,6 GlcNAc-branched N-glycans has major deleterious effects on E-cadherin-mediated cell-cell adhesion leading to the impairment of the stability and competence of the intracellular adhesive complex [26]. Concerning the cadherin-dependent adhesive interactions established between cadherin molecules, it was reported that the presence of β1,6 GlcNAc-branched N-glycans at the EC2 and EC3 subdomains of N-cadherin affects the potential of cis-dimerization [51, 52].

Furthermore, this study demonstrated that locking the specific N-glycosylationsite modified by GnT-V either by Asn-554 mutation or by silencing of GnT-V, resulted in a protective effect, by precluding the functional impairment of E-cadherin through the recovery of E-cadherin expression at the cell membrane, increased capacity of cis-dimerization and increased interaction with catenins. In accordance, Guo et al demonstrated that removal of β1,6 GlcNAc-branched N-glycans at N-cadherin extracellular domain resulted in an increased N-cadherin cis-dimerization capability [52].

The precise molecular mechanism by which N-glycans affects the molecular organization of E-cadherin-catenin complexes on the cytoplasmic side need to be carefully addressed. On the E-cadherin extracellular domains, N-glycans may interfere with the dimerization by steric hindrance leading to modulation of homotypic interactions, which consequently governs the dynamics of intracellular binding [53, 54]. Langer et al suggested that initial adhesion conferred by prevalence of cis dimers on cell membrane and modulated by N-glycosylation may alter the kinetics of cadherin-mediated intercellular binding. enhancing further additional cadherin interactions [51]. Taken together, these observations suggested that the β1,6 GlcNAc-branched N-glycans at E-cadherin Asn-554 may introduce conformational changes which reduced cis interactions decreasing the intracellular adhesive interactions. Nevertheless, removal of these specific N-glycans at E-cadherin Asn-554 by site –directed mutagenesis (E-cadherin M1) increased cis interactions that are followed by enhanced interaction with catenins.

Besides the contribution of β1,6 GlcNAc-branched N-glycans at E-cadherin Asn-554 on E-cadherin adhesive functions, the high mannose glycans present at E-cadherin Asn-633 may also play an important role on E-cadherin, more specifically on E-cadherin protein folding, as previously described [49]. Therefore, to achieve proper E-cadherin-mediated cell-cell adhesion, it is crucial the existence of high mannose glycans at E-cadherin Asn-633 and the absence of β1,6 GlcNAc-branched N-glycans at E-cadherin Asn-554. The impairment of this site-specific E-cadherin N-glycosylation pattern may affect the proper folding and the adhesive properties of E-cadherin. It is also important to note that E-cadherin M234, which display β1,6 GlcNAc-branched N-glycans at E-cadherin Asn-554 and absence

of high mannose N-glycans at E-cadherin Asn-633 exhibited a recovery of E-cadherin cellular localization and increased cis-dimerization after GnT-V silencing. This observation opens new questions about which mechanisms operate at Asn-554 that enable such recovery. Perhaps, action of other glycosyltransferases at each specific site may confer compensatory effects that lead to a proper N-glycosylation pattern of E-cadherin.

These *in vitro* results were further corroborated in the different *MGAT5* transgenic mouse models. *MGAT5* overexpressing mice display an abnormal pattern of E-cadherin cellular expression in gastric epithelial cells that overexpress β1,6 GlcNAc-branched N-glycans, resembling the mutant M234 or M4. In contrast, E-cadherin from *MGAT5* knockout mice, localized at the basolateral cell membrane, resembles the M1 mutant.

The present study contributed to understand how site-specific E-cadherin glycosylation modification can directly modulate E-cadherin-mediated cell-cell adhesion in cancer development and progression. Interestingly, from the clinical point of view, preventing E-cadherin site-specific glycosylation modifications was shown to improve its tumour suppressive functions in gastric cancer. This study contributes to the discovery of a promising glycobiomarker (site-specific) for improving early diagnosis of gastric cancer patients as well as for the development of targeted-specific therapeutic strategies.

Chapter IV – O-Mannosylation and N-glycosylation: two coordinated mechanisms regulating the critical functions of E-cadherin in cancer

E-cadherin was recently identified as a major target of O-mannosylation. Vester-Christensen et al. reported 37 members of the cadherin superfamily with O-Man glycosites [55]. The O-man-modified sites identified are situated in the EC domains EC2-5 of both classic type 1 and 2 cadherins and appear to display evolutionary conservation among cadherins. No O-Man-modified sites were identified in the EC1 domain of cadherins [55].

Concerning the biological functions of O-Man glycans on cadherin family, Lommel et al demonstrated that O-mannosylation of E-cadherin is required for the morula to blastocyst transition before implantation [56]. Prevention of O-mannosylation during the embryo development resulted in the arrest of morula-to-blastocyst transition process, being the absence of O-Man correlated with the disruption of E-cadherin at the adhesion sites. Furthermore, it was demonstrated that O-mannosylation is essential for E-cadherin-mediated cell adhesion in homeostasis. Moreover, it was showed that in a normal context, the O-linked mannose residues of E-cadherin are not elongated further [56]. Single O-mannosyl glycans were also reported in noncanonical heart cadherin from rabbit skeletal muscle [57]. Taking into consideration the fundamental role of glycosylation in regulating E-

cadherin functions in cancer, the understanding of how O-mannosylation contributes to the E-cadherin regulation in cancer is of utmost importance and remains unknown. The present work (chapter IV) aimed to unravel the role of O-mannosylation pattern in a gastric cancer context and its effects in the regulation of the biological functions of E-cadherin. Moreover, we aimed to elucidate the interplay between the two major types of E-cadherin post-translational modifications, N-glycosylation and O-mannosylation, and its relevance in cancer cell biology.

In the present work we demonstrated a reduced pattern of overall protein O-mannosylation in diffuse gastric cancer patients, concomitantly with an aberrant expression of E-cadherin into the cytoplasm. Furthermore, we showed that E-cadherin is highly O-mannosylated in a well differentiated context and its O-mannosylation profile remarkably decreases in a poorly-differentiated gastric cancer phenotype. Interestingly, poorly-differentiated cancer cells also display a decreased expression of POMT2 both at transcript and protein level. Concerning the biological functions of E-cadherin, the increased expression of O-mannosyl glycans on E-cadherin, by POMT2 overexpression, leads to a recovery of E-cadherin expression at the cell membrane in poorly-differentiated gastric cancer cell line, increasing the stability of the adherens junctions. In contrast, reduced expression of O-mannosyl glycans on E-cadherin, by POMT2 silencing, induces a mislocalization of E-cadherin and impairment of the assembly of the adhesion complex. Therefore, E-cadherin is O-mannosylated in homeostasis being crucial for E-cadherin-mediated cell-cell adhesion [56], and this post-translational modification is impaired during the acquisition of a malignant phenotype compromising the E-cadherin mediated adhesive properties.

Importantly, this work also provides new evidences about the interplay between O-mannosylation and GnT-V-mediated N-glycosylation in human gastric cancer patients. Neoplastic cells of human diffuse gastric carcinomas, characterized by a dysregulation of E-cadherin not explained by epi/genetic mechanisms, express high levels of β1,6 GlcNAc branched N-glycans and significant reduced levels of O-mannosylated proteins. The opposite was observed in human normal gastric mucosa displaying almost no expression of β1,6 GlcNAc branched N-glycans and high levels of expression of O-mannosylated proteins. Moreover, genetic manipulation of *MGAT5* gene in mice models revealled that *MGAT5* expression affects the expression of O-mannosylated proteins. This study provides for the first time direct evidences about the interplay of *MGAT5* expression and the O-mannosylation pathway. Furthermore, in an *in vitro* context, we showed that genetic manipulation of POMT2, either by POMT2 overexpression or silencing, has an impact on

GnT-V-mediated N-glycosylation, and particularly on E-cadherin. In turn, the overexpression of GnT-V induces a significant decrease of O-mannosyl glycans on E-cadherin. Taken together, a mechanistic interplay between O-mannosylation and GnT-V-mediated N-glycosylation pathways is herein postulated. In accordance, there is a competition between PMTs and OST enzyme complexes for the donor substrate [58, 59]. Moreover, O-mannosylation has been reported to precede the N-glycosylation process having the potential to alter the N-glycosylation site occupancy [60].

Concerning the site-specific E-cadherin glycosylation and taking into account the close proximity of the potential N-glycosylation and O-mannosylation sites of E-cadherin, we showed that preventing E-cadherin Asn-554 from receiving the β1,6 GlcNAc branched N-glycans structures results in an increased O-mannosylation profile of E-cadherin precluding its functional dysregulation and contributing to tumour suppression (chapter III + chapter IV), further supporting the existence of a coordinated interplay between O-mannosylation and N-glycosylation on the regulation of E-cadherin functions.

Under normal physiological circumstances, E-cadherin is primarily modified with high mannose/ hybrid N-glycans [53, 54], specifically on E-cadherin Asn-633 (chapter III), further undergoing O-mannosylation [56]. This specific glycosylation profile of E-cadherin contributes to a stable E-cadherin mediated cell-cell adhesion, essential in homeostasis. During malignant transformation, the glycosylation profile of E-cadherin undergoes significant changes [32]. E-cadherin exhibits complex branched N-glycans [33], specifically β1,6 GlcNAc branched N-glycans on E-cadherin Asn-554 (chapter III). Furthermore, taking into consideration the overexpression of *MGAT5* in a cancer context and having evidences that *MGAT5* affects the O-mannosylation pathway, the addition of O-Man residues to E-cadherin in the ER is impaired (chapter IV), and along the Golgi complex E-cadherin is predominantly modified by GnT-V- mediated N-glycosilation at Asn-554 (chapter III). The presence of β1,6 GlcNAc branched N-glycans on E-cadherin Asn-554 combined with the reduced levels of O-mannosyl glycans on E-cadherin impairs E-cadherin biological functions contributing to malignancy. Prevention of E-cadherin Asn-554 modification by GnT-V potentiates the O-mannosylation profile of E-cadherin associated with a recover of its functions. These two studies (chapter III and IV) enabled the disclosure of E-cadherin glycosignature in gastric cancer, regarding the N- and O-mannosyl glycans, which constitute promising glycobiomarkers for gastric cancer patients.

Concluding Remarks

The general aim of the present thesis was to contribute to the disclosure of the E-cadherin glycosignature in cancer and thus to contribute to the clinical explanation for those gastric carcinoma cases with E-cadherin dysfunction but without known epi/genetic alterations which encompasses the majority of the diffuse subtype gastric cancer. To achieve this general goal, we set specific aims from which resulted the following main conclusions.

1. The pattern of E-cadherin glycosylation undergoes significant alterations with implication for its biological functions, namely N-glycosylation mediated by GnT-III. E-cadherin has been reported to have an important role in the regulation of bisecting GlcNAc N-glycans expression, by a positive bidirectional crosstalk.

In this PhD thesis, we have found that exogenous E-cadherin expression inhibits IR, IGF-IR and ERK ½ phosphorylation. The activation of these signalling pathways results in E-cadherin mislocalization and decreased expression of bisecting GlcNAc N-glycans. Furthermore, insulin/IGF-I signalling induces a mesenchymal-like phenotype and increases the invasion potential of cancer cells. Altogether, our results provide new evidences about a novel networking player, insulin and IGF-I signalling, in the modulation of bisecting GlcNAc N-glycans expression on E-cadherin and its implications in cancer cell behaviour.

2. Human diffuse gastric cancer patients exhibited dysfunction of E-cadherin at least probably due to a markedly modification with β1,6 GlcNAc branched N-glycans, that contributes to the pathogenesis of gastric cancer.

In this doctoral thesis, we have characterized in detail the occupancy of the potential N-glycosylation sites of E-cadherin. Among the four potential N-glycosylation sites, only Asn-554 and Asn-633 were found to be occupied with complex and high mannose/hybrid-type N-glycans, respectively in a gastric cancer context. We have also identified that Asn-554 is the key site that is selectively modified with β1,6 GlcNAc branched N-glycans, catalysed by GnT-V. This site-specific glycosylation affects the E-cadherin biological functions in gastric cancer cells by interfering E-cadherin cellular localization, cis-dimer formation, assembly of the E-cadherin-catenin complex, and cell-cell aggregation capacity. Furthermore, we have shown that prevention of this deleterious glycan modification, either by site directed mutagenesis or GnT-V silencing, the E-cadherin biological functions are

recovered. Altogether, our results provide a functional and structural mapping of E-cadherin N-glycans in gastric cancer, contributing to the characterization of the role of each N-glycan (in a site-specific manner) in the regulation of E-cadherin biological functions in cancer.

3. Owing to the newly described O-mannosylation of E-cadherin in homeostasis, we further explored the role of O-mannosyl glycans on E-cadherin in a gastric cancer context and the interplay between O-mannosylation and GnT-V-mediated N-glycosylation.

We have shown that the overall protein O-mannosylation profile of human diffuse gastric cancer patients is remarkably reduced. Moreover, the E-cadherin O-mannosylation decreases during the acquisition of a poorly-differentiated gastric cancer phenotype, with effects in the impairment of its biological functions. Furthermore, we have verified that GnT-V-mediated N-glycosylation negatively affect protein O-mannosylation. Altogether, our results proposes the identification of O-mannosylation process as a novel mechanism of E-cadherin dysregulation in cancer that is precisely coordinated through the interplay between O-mannosylation and N-glycosylation machinery.

Future Perspectives

The present work provides evidences supporting that the dysregulation of E-cadherin in human gastric cancer is also explained by post-translational modifications through glycosylation. The pattern of E-cadherin glycosylation can be therefore considered as a potential clinical biomarker with promising applications to the early diagnosis, prognosis and also as appealing targets for the development of new therapeutic strategies in a gastric cancer setting. The identification of E-cadherin glycoprotein modified specifically with β1,6 GlcNAc branched N-glycans, being correlated with poorer survival (chapter III), followed by a remarkably reduction of protein O-mannosylation profile (chapter IV) could serve as a promising diagnostic and prognostic tool with utility in the clinical management of gastric cancer patients (from functional glycomics to clinical applications).

Furthermore, having identified the specific N-glycosylation site of E-cadherin modified with the deleterious N-glycans in a gastric cancer context, open the possibility for the production of (bi-functional) antibodies that specifically direct against Asn-554- β1,6 GlcNAc branched N-glycans- conjugated epitope, detecting specifically the deleterious E-cadherin glycoforms, envisioning a promise diagnostic tool. In addition, the disclosure of the precise E-cadherin glycopeptide responsible for the defective functions of E-cadherin in gastric cancer might also be viewed in future as an appealing therapeutic target, through preventing this site-specific N-glycan occupancy.

Interestingly from the clinical point of view is the fact that several reports have demonstrated that the extracellular domain of E-cadherin is frequently cleaved by proteases to generate a soluble ectodomain fragment which has been found to be increased in the serum of cancer patients. Considering that the E-cadherin glycoforms modified with β1,6 GlcNAc N-glycans predominate in gastric carcinoma, it would be very interesting to further explore the presence of such aberrant E-cadherin glycoform in the serum of cancer patients.

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Chapter VI

Gastric Cancer: adding glycosylation to the equation

Review

Cell PRESS

Gastric cancer: adding glycosylation to the equation

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Gastric cancer has a high incidence and mortality, so there is a pressing need to understand the underlying molecular mechanisms in order to discover novel biomarkers. Glycosylation alterations are frequent during gastric carcinogenesis and cancer progression. This review describes the role of glycans from the initial steps of the carcinogenesis process, in which *Helicobacter pylori* adheres to host mucosa glycans and modulates the glycophenotype, as well as how glycans interfere with epithelial cell adhesion by modulating epithelial cadherin functionality in gastric cancer progression. Other mechanisms regulating gastric cancer malignant behavior are discussed, such as increased sialylation interfering with key signaling pathways and integrin glycosylation leading to an invasive phenotype. Applications of these glycosylation alterations in the clinical management of gastric cancer patients are discussed.

Glycosylation in gastric cancer: understanding its functional roles

Cancer is a major cause of death worldwide, remaining an extremely important health problem [1]. Gastric cancer (GC) is the second leading cause of cancer-related death worldwide, affecting close to one million people per year [1].

Glycobiology has become a focus of research in cancer biology with several implications for the clinic. Glycans are major components of several biomolecules, including glycoproteins, glycosphingolipids, and proteoglycans expressed by cells and tissues. Glycans have been shown to be involved in various pathophysiological steps of tumor development and progression, regulating tumor cell proliferation, invasion, metastasis, and angiogenesis [2,3]. In the process of gastric carcinogenesis, glycans have been shown to be involved in various steps, such as, cell–pathogen interactions, cell–cell and cell–matrix interactions, cell differentiation, cancer cell migration, invasion and metastasis [2,4]. The understanding of glycans' role in these steps sets the ground for the development of novel cancer diagnostic and prognostic biomarkers, as well as novel pharmaceutical agents that target these molecules. Thus, deciphering the GC cell 'glycans code' will help to provide cutting-edge knowledge with potential applications for the improvement of GC clinical management.

This review provides a comprehensive view of the role of glycans in cancer with a particular focus on GC. We address the initial steps of the process of gastric carcinogenesis, in which *Helicobacter pylori* uses glycans to adhere and modulates the host gastric mucosa glycophenotype for chronic infection. We also present how glycans interfere with basic mechanisms involved in cell–cell adhesion, modulating the functionality of epithelial cadherin (E-cadherin), a key protein involved in the genesis and progression of gastric carcinoma. Furthermore, we present other major mechanisms that modulate the malignant behavior of gastric carcinoma, such as the glycosylation (see Glossary) of integrins and the expression of sialylated glycans, which interferes with key signaling pathways and leads to the acquisition of an invasive phenotype. Finally, we discuss important applications of glycosylation alterations as biomarkers to improve the clinical management of cancer patients.

Gastric cancer: epidemiology, clinicopathological features, and clinical management

GC represents a high burden in terms of incidence and cancer-related mortality, being the fourth most common malignancy in the world and the second leading cause of

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Glossary

Endoplasmic reticulum-associated degradation: is a protein degradation pathway in the endoplasmic reticulum that has a central clearance function in the cell. Is a cellular pathway that targets misfolded proteins and mutant proteins for ubiquitination and subsequent degradation by a protein-degrading complex called the proteosome.

Familial intestinal gastric cancer (FIGC): an intestinal-type gastric cancer syndrome with familial aggregation. The criteria of the International Gastric Cancer Linkage Consortium for FIGC vary accordingly with countries with high incidence of FIGC or low incidence of FIGC. The germline genetic defect underlying the disease remains unknown.

Glycosylation: a post- and co-translational modification process in which carbohydrates (glycans, saccharides, or sugars) are covalently attached to proteins, lipids, or other organic molecules. It is a tightly regulated process that occurs in the endoplasmic reticulum and Golgi compartment of all eukaryotic cells. This process depends on the availability of nucleotide sugar donors and on the coordinated action of a large number of enzymes (glycosyltransferases and glycosidases). The majority of mammalian proteins synthesized in the endoplasmic reticulum undergo glycosylation. There are two main classes of protein glycosylation: *N*- and *O*-glycosylation.

Hereditary diffuse gastric cancer (HDGC): an inherited form of diffuse-type gastric cancer that represents a rare but incurable autosomal-dominant gastric cancer syndrome with high penetrance. It is a highly invasive type of tumor with early onset gastric cancer (diagnosed before 45 years of age). Approximately 45% of families with HDGC have germline alterations in the E-cadherin (*CDH1*) gene. This early gastric cancer is located beneath an intact mucosal surface, and therefore early detection is extremely difficult. Prophylactic total gastrectomy is usually advised after the age of 20 and before the age of 40. In 1999, the International Gastric Cancer Linkage Consortium (IGCLC) proposed specific criteria to define HDGC. Families with aggregation of gastric cancer and an index case with diffuse IGCLC, but not fulfilling the IGCLC criteria for HDGC, are termed familial diffuse gastric cancer (FDGC).

Mucins: a family of glycoproteins with a high content of serine, threonine, and proline residues, and numerous *O*-linked glycans. Mucins can be membrane-bound or secreted onto mucosal surfaces. Mucins are high-molecular-weight and heavily glycosylated proteins constituting an important interface between many epithelial surfaces of the body and the exterior environment. The main characteristic of mucins is their ability to form gels.

***N*-glycosylation**: this is characterized by the addition of the glycan chains to an aspargine residue of the nascent protein in a consensus sequence Asn-X-Ser/Thr, where X can be any amino acid with the exception of proline. The resulting *N*-glycan structures are generally classified into three principal categories: high mannose, complex, and hybrid types (see Figure 3 in main text).

***O*-glycosylation**: a stepwise process characterized by the covalent linkage of glycan chains to the hydroxyl group of a serine or threonine. The initial step of *O*-glycosylation is controlled by a family of GalNAc transferases that transfer an initial *N*-acetylgalactosamine (GalNAc) residue. No consensus sequence defines an *O*-linked glycosylation site. The initial GalNAc can be extended, branched and elongated by several glycosyltransferases.

cancer death in both sexes worldwide [1,5]. The global incidence of GC has been declining over the past few decades, but the total number of cases is expected to rise as a result of the ageing population [5]. Most cases of GC are sporadic in nature, but approximately 10% display familial clustering and only 1–3% of these are predicted to be hereditary [6] (Figure 1).

Several GC classifications have been proposed over the past decades. The most commonly used are the World Health Organization and the Laurén classifications, the latter describing two main histological subtypes, intestinal and diffuse, that display different clinicopathological profiles and distinct epidemiological settings [7] (Figure 1).

The intestinal subtype of GC represents nearly 70% of the cases and predominates in high-risk areas, being more frequently observed in older male patients [8]. The carcinogenesis process leading to the intestinal subtype of GC is considered to be gradual and stepwise, leading to autonomously growing tumors with glandular structure [8]. The *H. pylori* infection is a pivotal risk factor for gastric carcinogenesis, with tumor development occurring only in a subset of individuals. The progression of the gastric carcinogenesis pathway has been shown to be related to the host characteristics, such as host genetic polymorphisms of pro-inflammatory interleukins [9,10] and molecules involved in adhesion of the bacteria [11–13], as well as genetic variations of the bacteria [such as differences in the *H. pylori* virulence factors cytotoxin-associated gene A (cagA) and vacuolating cytotoxin A (vacA)] [9] (Figure 1). Other risk factors have been described, such as diet [14] and familial aggregation [15]. Almost half of the world population is infected with *H. pylori*, and if occurring, GC is clinically diagnosed four or more decades later. During this period, a prolonged precancerous process takes place, represented by a 'cascade' of events with the following sequential histopathologic stages: chronic active non-atrophic gastritis, multifocal atrophic gastritis, intestinal metaplasia (IM) (precancerous conditions), dysplasia (precancerous lesion), and carcinoma [16] (Figure 1). The clinical guidelines for the management of these precancerous conditions and lesions on the stomach recommend a schedule for endoscopic follow-up of patients with extensive precancerous multifocal conditions [17,18]. Recently, a new hereditary syndrome has been identified, gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS), which is characterized by the autosomal dominant transmission of fundic gland polyposis, including areas of dysplasia or intestinal-type gastric adenocarcinoma restricted to the proximal stomach (Figure 1). Genetic defects behind this syndrome have not been elucidated yet [19].

Unlike the intestinal type of GC, the diffuse subtype (representing nearly 30% of GC cases) tends to occur in younger individuals, mainly females, and frequently depicts hereditary forms [20,21]. In contrast to the incidence of the intestinal subtype, which is steadily decreasing in most countries, the incidence of the diffuse subtype is stable [5]. Diffuse-type GC develops without the intermediate steps of glandular atrophy and IM. It is characterized by poorly cohesive cells, organized in cords or micro-glands, as well as discohesive cells that may widely invade the gastric wall, some of which may be of the signet ring cell type. Diffuse-type GC displays a major molecular abnormality: defective intercellular adhesion mainly resulting from E-cadherin dysregulation [22,23] (Figure 1). In most cases, this is due to abnormal expression of E-cadherin [an adhesion molecule encoded by the human cadherin 1, type 1, E-cadherin (*CDH1*) gene] [20,21,23]. Germline alterations (mostly mutations) of the *CDH1* gene are the genetic cause of hereditary diffuse gastric cancer (HDGC) [24].

In countries without screening programs for early detection of GC, most cases are diagnosed at an advanced stage. Most patients die during the first year after diagnosis, even if submitted to costly and aggressive therapy [25]. The five-year survival rates of GC remain extremely poor (around 10%) [26]. The diagnosis of GC at advanced disease stage increases the risk of relapse after surgical treatment. Consequently, GC remains a huge concern in clinical practice, and therefore new biomarkers and molecular tools are urgently needed in order to improve the success of early diagnosis, prognosis, and therapeutic options for GC patients and their relatives.

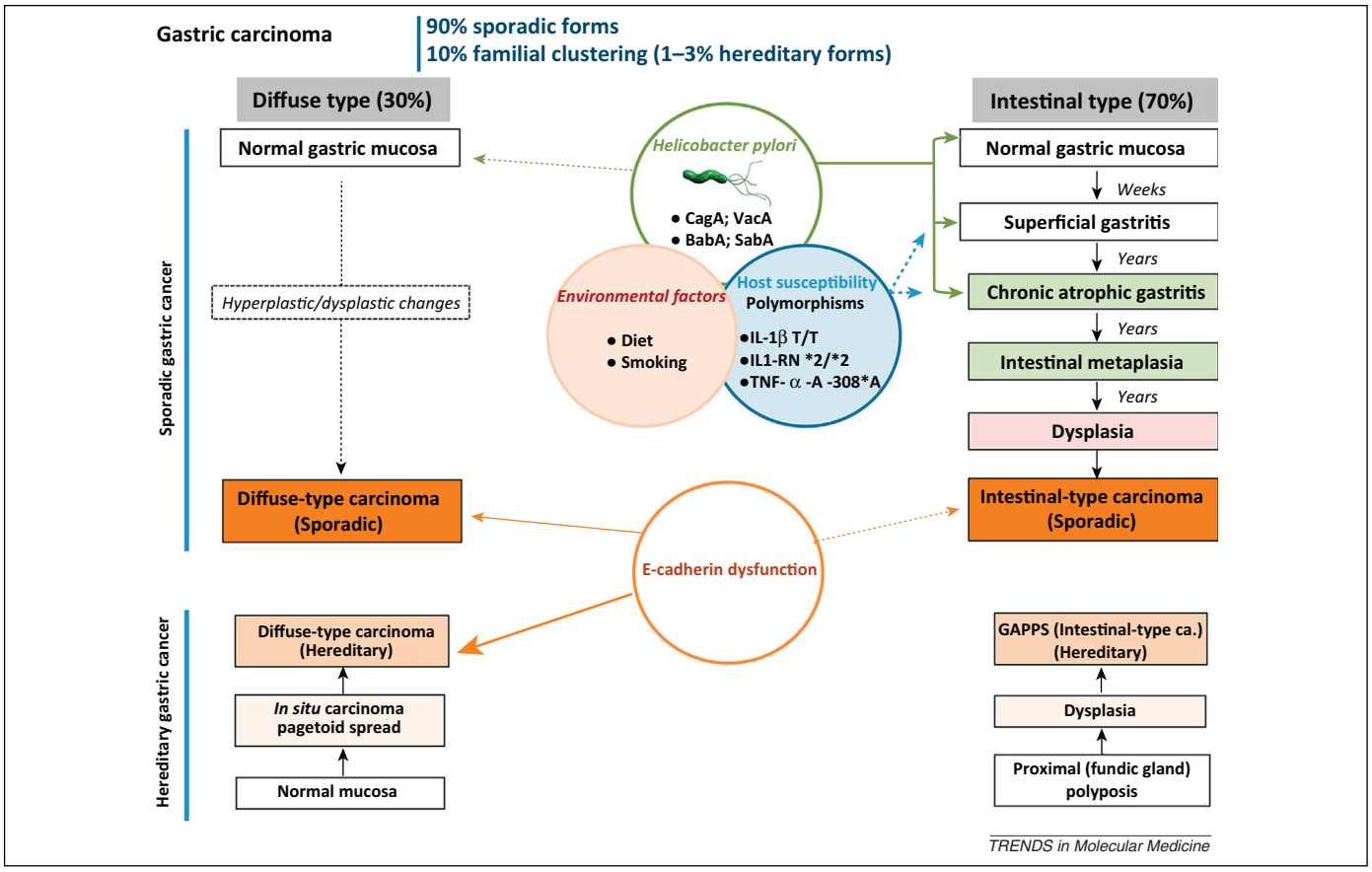

Figure 1. The two pathways of gastric cancer. There are two main histological subtypes of gastric cancer, the intestinal subtype (70% of cases) and the diffuse subtype (30% of cases), which display different clinicopathological profiles and occur in distinct epidemiological settings. Sporadic forms account for most cases (90%), whereas hereditary forms account for only 1–3% of cases. Abbreviations: BabA, blood group antigen-binding adhesin; GAPPS, gastric adenocarcinoma and proximal polyposis of the stomach; IL, interleukin; SabA, sialic acid-binding adhesin; TNF-α, tumor necrosis factor-α.

Glycosylation in normal gastric mucosa and gastric carcinogenesis

The gastric mucosa displays a set of mucins that are the most frequent carriers of *O*-glycans in higher eukaryotes [27]. In healthy gastric mucosa, the superficial foveolar cells express the membrane-associated mucin 1 (MUC1) and the secreted MUC5AC, which are the major components of the gastric mucus layer. The deeper gastric glands express the secreted MUC6 [28–30]. MUC5AC is accompanied by co-expression of type 1 Lewis a and Lewis b antigens, whereas MUC6 expression is associated with the presence of type 2 Lewis x and Lewis y antigens [30,31] (Figures 2 and 3).

Genetic polymorphisms in glycosyltransferase genes lead to different expression profiles of gastric mucosa histo-bood group antigens. For instance, the fucosylated H-type 1 antigen is only expressed in the foveolar epithelium of secretor individuals and the difucosylated Lewis b antigen is only expressed in secretor and Lewis-positive individuals [32]. Secretor individuals constitute 80% of the Caucasian population and express a functional secretor fucosyltransferase 2 (FUT2) enzyme; Lewis-positive individuals represent 90% of the Caucasian population and express the Lewis FUT3 enzyme [33]. Genetic polymorphisms of the genes encoding these enzymes are associated with different host susceptibilities to infection by *H. pylori* [11,34].

H. pylori is a major trigger of gastric carcinogenesis, and the colonization of the gastric mucosa depends on bacterial attachment to the gastric mucus layer and epithelial cells. This binding is mediated by bacterial outer-membrane proteins, named adhesins, which specifically bind to host glycosylated receptors (Figure 2). Blood group antigen-binding adhesin (BabA) binds to the fucosylated blood group antigens H-type 1 and Lewis b [35,36], which are present in the MUC5AC mucin of gastric epithelial cells of healthy secretor individuals. Some *H. pylori* strains, named generalists, admit modification of Lewis b with AB glycan determinants, whereas specialist strains only recognize the naked form of this epitope [37]. BabA was also shown to bind Globo H and Globo A, which are blood group O and A determinants, respectively, on type 4 core chains [38]. Individuals infected with strains that harbor a functional BabA adhesin present a higher risk of developing more severe gastric lesions, including IM and gastric adenocarcinoma [39].

The gastric glycosylation patterns define the *H. pylori* tropism, and bacteria are mainly found at the surface of mucous cells, where there is co-expression of MUC5AC and type 1 fucosylated Lewis antigens. By contrast, colonization of the deeper gastric glands where MUC6 is expressed is rare. This distribution is explained by the presence of unique *O*-glycan structures with terminal α1,4-linked *N*-acetylglucosamine (α1,4GlcNAc) residues in the MUC6

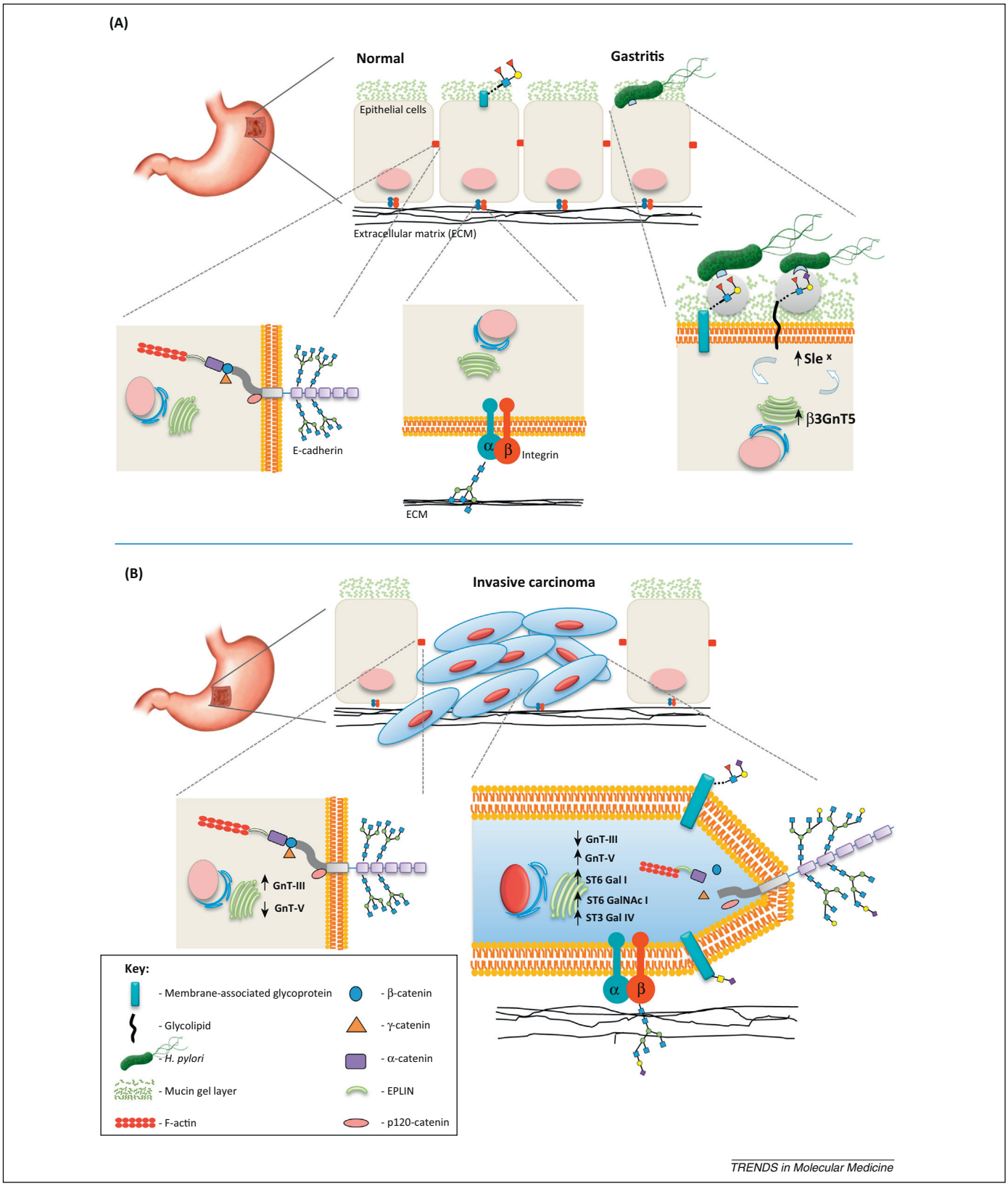

Figure 2. Schematic representation of the glycosylation phenotype in normal gastric mucosa and gastritis **(A)**, and the glycosylation alterations in gastric cancer cells **(B)**. (a) In normal gastric mucosa, the epithelial cells display normal cell–cell adhesion mediated by epithelial cadherin (E-cadherin) glycosylated with stabilizing glycoforms: the bisecting *N*-acetylglucosamine (GlcNAc) *N*-glycan structures. The cell–extracellular matrix (ECM) interaction is mediated by integrins that are also modified with bisecting GlcNAc glycan structures. The mucin gel layer covering the gastric mucosa plays an important role in the colonization of *Helicobacter pylori*. Normal gastric cells express fucosylated glycans such as Lewis b antigen, which serves as a ligand *for H. pylori* adhesion and infection, leading to the gastritis process. *H. pylori* binding is mediated by bacterial adhesins, such as blood group antigen-binding adhesin (BabA), which specifically binds to Lewis b, and sialic acid-binding adhesin (SabA), which binds to sialyl-Lewis x antigens expressed during inflammation of the gastric mucosa. *H. pylori* has been shown to induce an overexpression of β3GnT5, leading to increased biosynthesis of sialyl-Lewis x. (b) During the gastric carcinogenesis process, the gastric epithelial cells undergo several changes in glycosylation that affect the malignant behavior of cancer cells. E-cadherin expression induces *N*-acetylglucosaminyltransferase-III (GnT-III)-mediated glycosylation, which confers advantages over GnT-V-mediated

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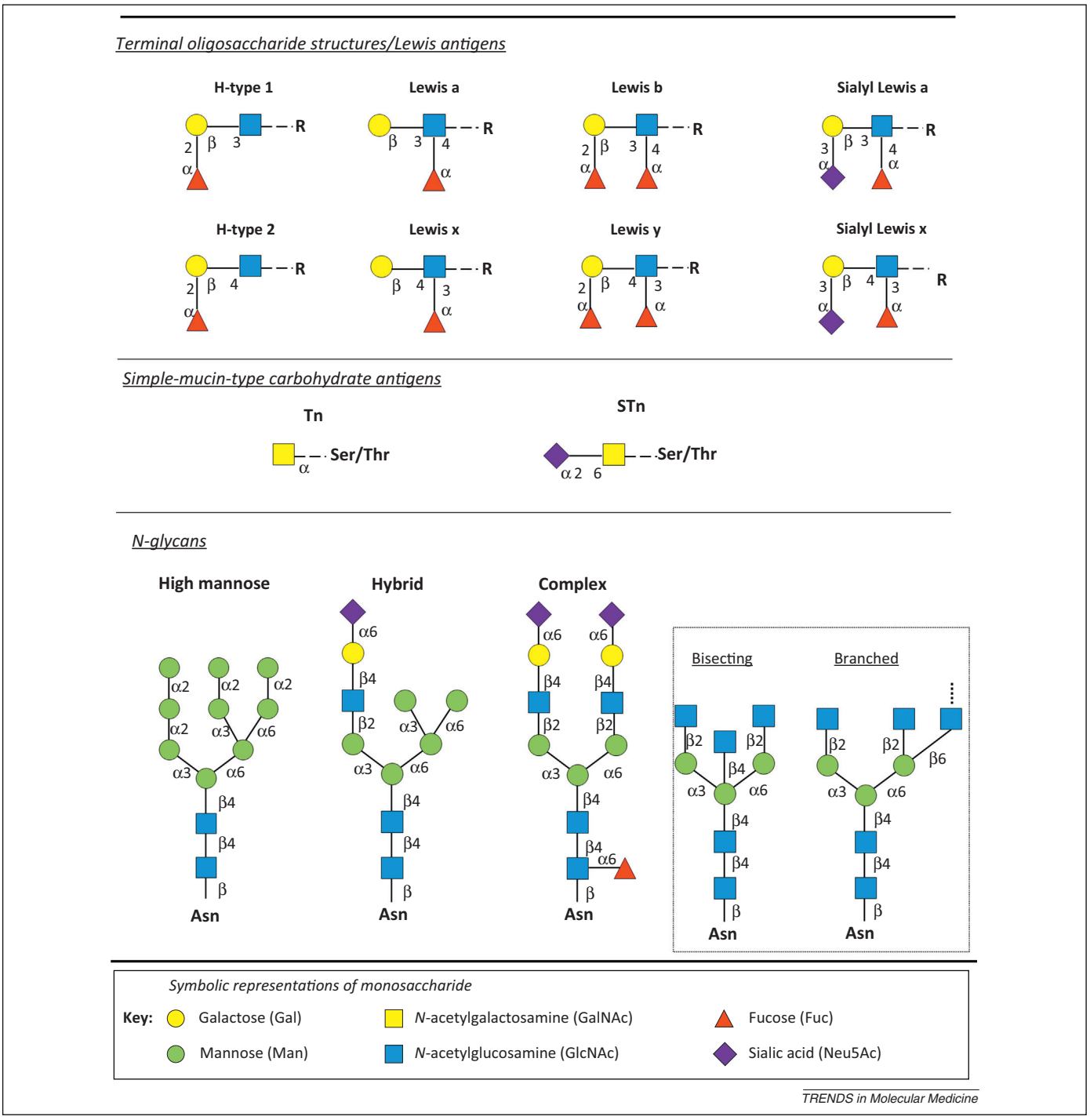

Figure 3. Summary of important glycan structures expressed in gastric tissues. Schematic representation of the most common glycans structures expressed in gastric tissues either in normal or in pathological conditions. These glycan structures include terminal Lewis and sialylated Lewis structures; simple mucin type glycan structures, such as Tn and sialyl-Tn; and the *N*-glycans, particularly the complex types: bisecting *N*-acetylglucosamine (GlcNAc) and the β1,6GlcNAc branched structures. Abbreviation: R, core oligosaccharide.

backbone. Terminal α1,4GlcNAc presents a natural antimicrobial activity by inhibiting the synthesis of α-glucosyl cholesterol, an important constituent of the *H. pylori* cell wall, and therefore suppressing bacterial growth [40]. In addition, α1,4GlcNAc expression was recently shown to suppress tumor-promoting inflammation. Mice that lack the enzyme responsible for α1,4GlcNAc biosynthesis (*A4gnt*$^{-/-}$ mice) develop gastric adenocarcinoma in the absence of *H. pylori* infection, demonstrating that α1,4GlcNAc loss is sufficient for cancer initiation in this model [41].

glycosylation. The modification of E-cadherin and integrins with bisecting GlcNAc structures is associated with suppression of tumor cell invasion. On the contrary, the modification of E-cadherin with β1,6GlcNAc branched structures promotes dysfunction of E-cadherin-mediated cell–cell adhesion, leading to an invasive phenotype. This malignant phenotype is also due to an increased modification of integrins with the branched *N*-glycan structures that leads to an increased interaction with the ECM. The overexpression of sialylated glycan structures, such as sialyl-Lewis x and sialyl-Tn, has also been shown to contribute to the aggressive phenotype of gastric tumor cells. Changes in the expression of sialyltransferases and an induction of sialyl-Lewis x overexpression in gastric cancer cells alters the activation of signaling pathways, leading to an invasive phenotype. Abbreviations: EPLIN, epithelial protein lost in neoplasm; β3GnT5, β3-N-acetylglucosaminyltransferase-5.

Box 1. Genetic/epigenetic regulation of E-cadherin expression in gastric cancer

Loss or abnormal expression of the epithelial cadherin (E-cadherin) gene [cadherin 1, type 1, E-cadherin (*CDH1*)] and protein in neoplastic cells generally causes them to turn into the invasive components of several human cancers, including gastric cancer (GC) [60]. Hereditary diffuse gastric cancer (HDGC) is the archetypical example of E-cadherin loss significance and is caused by heterozygous germline *CDH1* point mutations in about 40% and large deletions in ~4% of all families with this syndrome [24,136]. In the sporadic context, a recent comprehensive analysis showed that *CDH1* somatic structural alterations were found in ~10% of all GC cases: 10.9% within the sporadic setting and 9.7% among familial cases [23]. Importantly, the worst patient survival rate among all GCs analyzed was seen in patients with tumors carrying *CDH1* structural alterations belonging to familial intestinal gastric cancer (FIGC) families [23].

In all GCs, epigenetic silencing through *CDH1* promoter hypermethylation has been described, with frequencies varying from 50% to 83% in diffuse tumors and from 6.25% to 50% in intestinal tumors [137,138]. Recently, a large GC study revealed *CDH1* somatic hypermethylation in 20.7% of GC cases: 18.4% of the cases within the sporadic setting and 26.4% within the familial setting. Particularly among HDGC patients carrying germline *CDH1* mutations, the most frequent somatic event affecting the *CDH1* gene is promoter methylation that leads to complete somatic inactivation of the *CDH1* gene [either as a single event (~50%) or concomitantly with loss of heterozygosity at the *CDH1* locus (~19%)] [139,140]. Primary HDGC tumors that lack germline *CDH1* mutations show exclusively *CDH1* promoter hypermethylation in 50% of the cases. Among patients with FIGC, 17.0% of tumors showed hypermethylation [23].

MicroRNAs (miRNAs) have also emerged as a new layer of *CDH1* gene regulation [141]. Members of the miR-200 family of miRNAs have been implicated in the regulation of E-cadherin expression through direct targeting of the E-cadherin transcriptional repressors zinc finger E-box-binding homeobox 1 (ZEB1) and ZEB2. Inhibition of endogenous miR-200 expression levels led to a relief of ZEB1 and ZEB2 repression, a reduction of E-cadherin mRNA expression levels, and, consequently, a mesenchymal-like morphology and increased cell migration [142]. Apart from the miR-200 family, miR-9 and miR-101 have also been implicated in the network of E-cadherin regulation [137]. miR-101 has been shown to be downregulated in GC in comparison with normal gastric mucosas and, at least in 65% of GC cases, this downregulation was due to deletions and/or microdeletions at miR-101 genomic loci.

In healthy conditions, gastric mucosa expresses mostly neutral fucosylated glycans, but *H. pylori* infection and the associated host inflammatory response can induce a remodeling of the gastric glycophenotype with *de novo* expression of sialylated antigens, including sialyl-Lewis x and sialyl-Lewis a. *H. pylori* has been shown to induce an overexpression of β3-N-acetylglucosaminyltransferase-5 (β3GnT5), leading to increased biosynthesis of sialyl-Lewis x [42]. *H. pylori* binding to inflamed tissue is mainly mediated by sialic acid-binding adhesin (SabA) [43].

During gastric carcinogenesis, aberrant cell-surface glycosylation is frequently observed in precancerous conditions, such as IM, which shows major alterations of mucin expression with marked *de novo* expression of the intestinal MUC2 mucin [44] and aberrant expression of the simple mucin-type antigen sialyl-Tn (Figure 3) in goblet cells [45,46]. Recently, plasminogen with sialyl-Tn was identified in the serum of patients with gastritis and IM, suggesting its possible application as a biomarker for non-invasive clinical screening and diagnosis [47].

In GC, various types of glycosylation alterations have been described and shown to play key roles in modulating cancer cell behavior (Figure 2). These molecular mechanisms are discussed in detail in the following sections.

Cellular adhesion in homeostasis and gastric cancer: E-cadherin as a key molecule

Cellular adhesion determines the polarity of cells, participating in the spatial and functional tissue organization of epithelial cells [48,49]. Cell–cell and cell–matrix adhesion are involved in various processes of tissue morphogenesis and in the maintenance of mechanical integrity and normal cell physiology [50]. Integrins and E-cadherin are fundamental adhesion molecules with key roles in cell–extracellular matrix (ECM) and cell–cell interactions, respectively, being essential for normal tissue architecture. Alterations of glycosylation in these key molecules interfere dramatically with their functions and can modulate physiological and pathological processes.

One of the major regulators of epithelial tissue morphogenesis and homeostasis is the cell–cell adhesion molecule E-cadherin [51,52]. The critical importance of E-cadherin in normal development is demonstrated by the lethality of E-cadherin knockout mice at an early stage in embryogenesis [53].

In the process of carcinogenesis, alterations in cell–cell and cell–matrix adhesion have a central role [50,54,55]. The downregulation or inactivation of E-cadherin-mediated cell–cell adhesion may occur in early steps of cancer development [55–57]. Specifically, in diffuse-type GC, E-cadherin dysfunction occurs in intra-epithelial pre-invasive lesions, such as *in situ* carcinoma and pagetoid spread of signet ring cells in the setting of HDGC (caused by *CDH1* germline mutations) [20,58] (Figure 1). These lesions display decreased or absent expression of E-cadherin. Similar pre-invasive lesions of GC have not been identified in the sporadic setting. In the intestinal subtype of GC, alterations in E-cadherin expression occur predominantly at later stages of cancer progression (Figure 1). These alterations in E-cadherin expression have been associated with poor clinical outcome of GC patients [23,59].

Several molecular mechanisms explain the abnormal E-cadherin expression in GC at the genetic and epigenetic level (Box 1), and also at the post-translational level through glycosylation.

The mature E-cadherin protein contains a single transmembrane domain, a cytoplasmic domain, and an extracellular domain, which comprises five repeated subdomains (EC1–EC5) [60]. The extracellular domain mediates the Ca^{2+}-dependent homophilic/homotypic cell–cell adhesion. The E-cadherin ectodomain has four potential *N*-glycosylation sites: two in EC4 (Asn554 and Asn566) and two in EC5 (Asn618 and Asn633). These *N*-glycan sites were found to be crucial for E-cadherin folding, expression, and biological functions [61,62]. Cell–cell adhesion is further accomplished through the molecular interaction between the E-cadherin cytoplasmic domain and catenins (β-catenin, γ-catenin, p120 catenin, and α-catenin), which couple cadherin to the actin cytoskeleton [60]. The interaction with p120

catenin has been shown to regulate E-cadherin internalization, preventing its endocytosis and promoting its stability at the cell surface [63]. E-cadherin–catenin complex is the major component of the adherens junctions (AJs), and its stability is regulated by E-cadherin glycosylation [64]. Furthermore, the engagement of E-cadherin in homophilic interactions can act as a mechanosensor, triggering the activation of signal transduction pathways that are important in tissue organization [65,66] and in which glycosylation modifications play a role.

Glycosylation as a modulator of gastric cancer cellular behavior

Role of glycosylation as a regulator of E-cadherin-mediated gastric tumor development and progression

Although epigenetic and structural alterations of E-cadherin may be considered instrumental for protein downregulation or inactivation (Box 1), cancer cells have been shown to exhibit additional mechanisms that contribute to the acquisition of the malignant phenotype.

In GC, E-cadherin immunostaining does not always indicate the absence of expression (which is compatible with epigenetic or structural alterations), but frequently shows a redistribution from the cell membrane into the cytoplasm (aberrant E-cadherin expression). Around 70% of all GC cases showing E-cadherin aberrant expression do not harbor *CDH1* structural alterations (mutations, loss of heterozygosity, or hypermethylation) [23] (Box 1).

Recent evidence has shown that glycosylation can interfere with E-cadherin functions in normal and pathological conditions. E-cadherin *N*-glycosylation seems to be essential for its correct folding and transport to the cell surface [62]. Elimination of *N*-glycans at Asn633 was demonstrated to affect E-cadherin expression, targeting it for endoplasmic reticulum-associated degradation [62]. The regulation of the recycling and trafficking pathways of E-cadherin has been demonstrated to be controlled by glycosylation [67,68]. Cytoplasmic *O*-glycosylation of E-cadherin (*O*-GlcNAc) was shown to block its cell surface transport, precluding binding to p120 catenin, which results in reduced intercellular adhesion [69,70]. *N*-glycosylation of E-cadherin has also been reported to affect E-cadherin-mediated signaling pathways, particularly the activation of the extracellular-regulated kinase pathway [71] (which has also been described for neural cadherin [72]), suggesting that glycosylation may influence the mechanotransduction role of E-cadherin. Moreover, the E-cadherin *N*-glycans have been shown to affect E-cadherin's intercellular adhesive functions [73] by interfering with the molecular assembly and maturity of the AJ, and consequently the assembly of tight junctions [61,74–76].

During malignant transformation, E-cadherin displays substantial alterations of its glycans [77] (Figure 2). E-cadherin controls its own glycosylation by promoting expression of *N*-acetylglucosaminyltransferase-III (GnT-III), which confers a stabilizing E-cadherin glycosylation at the cell membrane, increasing cell–cell adhesion [78,79] (Figure 2). This beneficial glycosylation of E-cadherin with bisecting GlcNAc *N*-glycans, catalyzed by GnT-III, is associated with a delayed turnover of E-cadherin at the cell surface and an inhibition of E-cadherin endocytosis [64,80]. E-cadherin modified with bisecting GlcNAc *N*-glycans contributes to the increased recruitment of catenins and increased AJ stability. This glycosylation type of E-cadherin promotes increased intercellular adhesion and a downregulation of intracellular signaling pathways involved in cell motility [81], supporting its contribution to tumor suppression (Figure 2). In addition, this E-cadherin *N*-glycoform also contributes an epithelial phenotype that prevents the epithelial-to-mesenchymal transition (EMT) process [82,83]. The modification of E-cadherin with bisecting *N*-glycoforms was found to have an important role in the suppression of human gastric carcinoma progression [64].

Conversely, when E-cadherin is glycosylated by GnT-V, there are major effects on the dysregulation of its functions in a GC context. GnT-V is known to be upregulated in gastric carcinoma [84], leading to biosynthesis of the β1,6GlcNAc branched *N*-glycans and contributing to cancer cell invasion and metastases [85,86]. GnT-V overexpression in a GC cell line model induces significant alterations on E-cadherin cellular expression, with a delocalization from the cell membrane into the cytoplasm (aberrant E-cadherin expression) [64]. Concomitantly with this, GC cells acquire a fibroblastoid/mesenchymal appearance compatible with an EMT phenotype induced by GnT-V [87]. In addition, GnT-V-mediated glycosylation on E-cadherin has been shown to interfere with the molecular assembly and stability of AJ. The β1,6GlcNAc branched *N*-glycans on E-cadherin lead to an impairment of β-catenin and p120 catenin recruitment, disturbing the stability of AJs, which affects the cell–cell adhesion capability of gastric tumor cells [64] (Figure 2). Moreover, β1,6GlcNAc branched *N*-glycans on cadherins can also affect the tyrosine phosphorylation of catenins associated with increased cell migration and invasion [88,89]. GC cell line models overexpressing GnT-V showed an increased metastatic capability when injected in athymic nude mice [90]. In the clinical setting, gastric carcinoma cells (diffuse type) exhibiting an aberrant E-cadherin expression without E-cadherin epigenetic or structural alterations showed an increased modification with β1,6GlcNAc branched structures [64].

In summary, other than epigenetic and structural alterations, modification of the glycosylation of E-cadherin can remarkably disturb its normal function by promoting tumor cell development and progression, constituting a potential biomarker for clinical applications.

Integrins and gastric cancer: role of glycan modifications in integrin-mediated cell spreading and migration

Integrins are the main link between a cell and the ECM, playing essential roles in cancer invasion and metastases. They consist of α- and β-subunits. Each subunit has a large extracellular region, a single transmembrane domain and a short cytoplasmic tail (except for β4 integrin). The most general feature of integrins is that their interaction with their ligand can activate intracellular signaling pathways and cytoskeletal formation (outside-in signaling). Another important feature of integrins is inside-out signaling, in which intracellular signals received by integrins or other receptors in turn activate their extracellular domain and contribute to the assembly of the ECM [91,92]. Therefore glycosylation modifications of integrins play major roles in

the interaction between the cell and the ECM by interfering with biological functions, including cell spreading, migration, and signal transduction.

Integrins are glycoproteins and major carriers of *N*-glycans. α5β1 integrin, which is one of the best-characterized integrins, is modulated with *N*-glycans that are required for αβ heterodimer formation and integrin–matrix interactions [93,94]. Indeed, the integrin cannot bind to its substrate or be normally transported to the cell surface in the presence of the *N*-glycosylation inhibitor tunicamycin and deglycosylation mutants [95–97]. Additionally, alterations on *N*-glycans of α5β1 integrin could significantly contribute to tumor formation, invasion, and metastases. Cells transformed with the oncogenic Ras gene showed an enhanced cell spreading on fibronectin (FN) owing to an increase of β1,6GlcNAc branched glycans in α5β1 integrins [98]. Similarly, the characterization of carbohydrate moieties of α3β1 integrin from non-metastatic and metastatic human melanoma cell lines showed that β1,6GlcNAc branched structures were highly expressed on α3β1 integrin from metastatic cells compared with α3β1 integrin from non-metastatic cells [99]. In a GC context, overexpression of GnT-V leads to severe peritoneal dissemination in athymic mice and increased cellular migration owing to the increased expression of matriptase [90]; as well as due to alterations on E-cadherin-mediated cell–cell adhesion [64] (as discussed above), but also through the specific modification of α3β1 integrin with β1,6GlcNAc-branched *N*-glycans, which promotes increased cell migration [89] (Figure 2).

In contrast to GnT-V, the overexpression of GnT-III in GC cells inhibited α3β1 integrin-mediated cell migration by directly counteracting the effect of GnT-V-mediated glycosylation on α3β1 integrin [89] (Figure 2). As a result, GnT-III inhibits GnT-V-induced cell migration in GC cells. Two mechanisms have been proposed for the inhibition of cell motility and invasion in GC cells on overexpression of GnT-III: an enhancement in E-cadherin-mediated cell–cell adhesion [64] (as discussed above) and the downregulation of integrin-mediated cell–ECM adhesion [89]. These results indicate that GnT-III counteracts GnT-V and strongly suggest that remodeling of glycosyltransferase-modified *N*-glycan structures either positively or negatively modulates cell adhesion and migration in a GC context [64,73,100,101].

Consistently, overexpression of GnT-III resulted in an inhibition of α5β1 integrin-mediated cell spreading and migration, and the phosphorylation of the focal adhesion kinase [102]. The affinity of the binding of integrin α5β1 to FN was significantly reduced as a result of the introduction of a bisecting GlcNAc to a specific *N*-glycosylation site on the α5 subunit [103].

Overall these observations suggest that glycosylation has a critical role in GC through modulation of key pathophysiological steps in gastric tumor genesis and progression, such as cell–cell and cell–ECM interactions.

Sialylated glycans and gastric cancer: modulating tumor cell signaling and behavior

GC cells frequently display high levels of terminal sialylated glycans such as sialyl-Lewis x, which has been associated with venous invasion and poor disease prognosis [104] (Figure 2). Recently, the overexpression of sialyl-Lewis x in cell lines transfected with ST3 β-galactoside α-2,3-sialyltransferase 4 (ST3GAL4) was shown to increase the cells invasive potential both *in vitro* and *in vivo* [105]. This alteration of cell behavior was shown to be mediated by the activation of c-Met receptor tyrosine kinase and its downstream targets, such as focal adhesion kinase and Src proteins, as well as the cell division control protein 42 (Cdc42), Rac1 and RhoA GTPases [105]. These results demonstrate that the expression of sialyl-Lewis x in membrane-associated and secreted glycoconjugates of GC cells can cause major alterations in receptor tyrosine kinases and intracellular signaling pathways controlling epithelial cell invasion behavior, and therefore they play a key role in the aggressiveness of GC cells.

Another sialylated glycan frequently expressed in GC is the sialyl-Tn antigen [106] (Figure 2), which is recognized as an indicator of poor prognosis [107,108]. ST6GalNAc-I is the major enzyme controlling the expression of sialyl-Tn antigen in GC [109,110]. Sialyl-Tn expression in GC cells has been shown to induce major morphological and cell behavior alterations, including decreased cell–cell aggregation, altered ECM adhesion, and increased migration and invasion *in vitro* [111]. These data indicate that the sialyl-Tn antigen is able to modulate a malignant phenotype, inducing a more aggressive cell behavior.

Overall the alterations of expression of terminal sialylated antigens underlie key molecular events associated with gastric tumor cell–cell and cell–matrix interactions, signaling activation, migration, invasion, and metastases.

Glycosylation as a tool for the clinical management of cancer patients

One of the major concerns of cancer clinical management (and GC in particular) is to improve the early diagnosis and the successful rate of the therapeutic strategies. New approaches for cancer early diagnosis and treatment, and new biomarkers for risk stratification are urgently needed, and glycans can be a source for such applications (Box 2).

Currently, most of the traditional cancer serological markers, such as CEA and CA19-9 (for GC), CA125 (for ovarian cancer), and CA15-3 (for breast cancer), are based on detection of glycoconjugates (glycoproteins and glycolipids) shed from the tumor cells into the bloodstream [112–114].

Box 2. Outstanding questions

- Could specific glycosylation alterations be a source of novel biomarkers for improving the clinical practice in oncology? Are they sensitive and specific for the cancer condition?
- Could the specific detection of glycoforms on key proteins in serum and/or tissues contribute to the early diagnosis and determination of prognosis of cancer patients? Can we easily detect the glycomarkers during the routine screening of patients, such as in gastric biopsies and/or in blood samples?
- How can we use tumor-associated glycans to improve cancer therapy? Can we specifically modify glycan biosynthesis in cancer cells? Can we modulate the activity of glycosyltranferases and the biosynthesis of tumor-associated glycans to control aggressive cancer cell behavior? Can we use proteins bearing tumor-associated glycoforms as a target for immunotherapy approaches?

However, the reduced specificity and sensitivity of these serological assays for early detection of cancer is driving a search for novel biomarkers. In fact, the detection of specific glycoforms of a certain protein could contribute to the establishment of a biomarker with higher specificity for early detection of cancer or for diagnosis at a precancerous stage [115–117]. For example, fucosylated α-fetoprotein (L3 fraction) was approved by the US Food and Drug Administration as a marker for early detection of hepatocellular carcinoma (HCC); it appears in serum at the stage of liver cirrhosis just before the onset of HCC, being therefore considered the best approved marker in patients with HCC [118,119]. Recently, altered *O*-glycosylation (sialyl-Tn antigen) has been detected in circulating serum plasminogen in patients with IM and gastric carcinoma [47]. Such alterations detected in early stages of the carcinogenesis process might have valuable applications in the early diagnosis setting. In addition, recent reports have proposed fucosylated haptoglobin as a novel biomarker for pancreatic and colon cancer [120,121].

In line with this, the specific modification of E-cadherin or integrins with the deleterious β1,6GlcNAc branched structure might also be considered a potential biomarker for selecting at-risk patients for clinical surveillance. It is tempting to suggest the assessment of the pattern of E-cadherin/integrin glycosylation in a gastric biopsy sample as a biomarker for the early diagnosis and prognosis of GC. Furthermore, in combination with the current diagnostic procedures, the clinical search of specific glycoforms in key proteins, both in tissues and/or in serum, is of utmost importance to improve early diagnosis, determination of prognosis, and risk stratification of cancer patients (Figure 4). Several studies are now being conducted in order to assess aberrant glycoforms of specific proteins as candidate glyco-markers for improving clinical practice in oncology. In addition, the terminal carbohydrate determinants sialyl-Lewis x and Sda (a blood group carbohydrate antigen) on specific proteins may constitute interesting markers in the clinical setting, particularly as biomarkers for cancer cell biological behavior [122].

Glycosylation modifications are also good targets for cancer therapy. In the case of GC, targeting deleterious glycosylation pathways, such as synthesis of the β1,6GlcNAc branched *N*-glycans or terminal sialylation with sialyl-Lewis x, appears to be a promising approach with potential treatment applications. Inhibition of the biosynthesis of specific glycan structures can be achieved using synthetic compounds, and these are attractive tools for modulation of cancer cell behavior [123,124]. Swainsonine, an inhibitor of the Golgi protein α-mannosidase II, which blocks the synthesis of complex type *N*-glycans, has been demonstrated to have antitumor properties *in vitro* [125], *in vivo* [126], and in patients with advanced malignancies [127]. However, its efficacy in the clinical setting has been shown to be limited [128]. Additional specific compounds targeting specific glycosyltransferases expressed in specific cells might constitute a source of important tools for modulating cancer cell behavior contributing to cancer therapy.

Furthermore, anticancer vaccines targeting tumor-associated carbohydrate antigens provide another appealing option for cancer treatment; these have major advantages, as they can be designed to incorporate only those elements required for a desired immune response [129–131]. Various studies have shown that passive immunotherapy using antibodies directed to glycoform-specific targets, such as MUC1 mucin, expressed in tumor cells can be effective in inducing an antibody-dependent cell-mediated cytotoxicity [132]. Moreover, various active immunotherapy studies targeting glycoproteins expressed in tumor cells have been tested and are under clinical evaluation [133].

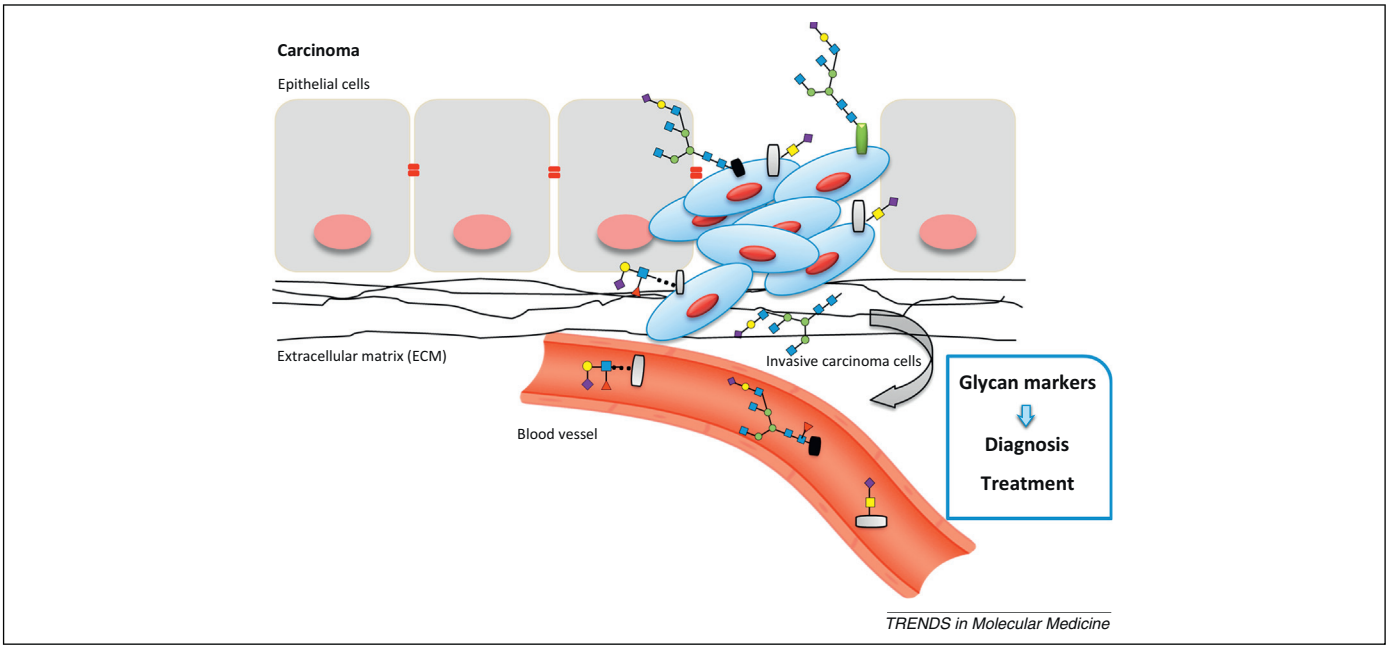

Figure 4. Glycans as potential biomarkers in the clinical setting. The alteration of cellular polarity and topology of cancer cells can lead to the shedding of glycoconjugates bearing important tumor-associated glycoforms into the bloodstream. These might constitute valuable molecular markers that can be detected in serological assays and used to improve the early diagnosis, prognosis, risk stratification, and surveillance of cancer patients.

In the GC setting, we can envision the design of glycopeptides based on proteins expressed by gastric carcinoma cells, such as MUC1, E-cadherin, or integrins specifically modified with cancer-associated glycans, as attractive compounds that are able to induce a classical major histocompatibility complex-mediated immune response [129]. Such glycopeptides could be a promising anticancer vaccine for GC treatment. The identification of the specific sites of glycosylation and cancer-associated glycoforms of key proteins expressed by GC remains of utmost importance and will aid in the effective design of synthetic glycopeptides for anticancer vaccine development.

Concluding remarks and future perspectives

The characterization of glycosylation in a given cell or tissue context is crucial for the understanding of its role in physiologic and pathologic conditions. In gastric tissues, glycans mediate the adhesion and infection of *H. pylori* as well as gastric carcinogenesis, and the alterations of *O*-linked and *N*-linked glycans modulate key mechanisms of GC progression, such as cancer cell invasion and metastasis.

Our comprehension of cancer cell biology will be improved through understanding the molecular mechanisms that control glycosylation alterations in these disease states — such as determining the glycosyltransferases involved in such changes — and through the constant technological improvement of the analysis of glycan structure in key proteins. Such progress will provide markers for improving the early diagnosis, prognosis, risk stratification, and surveillance of cancer, as well as the development of novel therapeutic strategies that will ultimately benefit cancer patients [114,134,135].

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Disclaimer statement

The authors declare no conflicts of interest.

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Appendix

Appendix I

Insulin/IGF-I Signaling Pathways Enhances Tumor Cell Invasion through Bisecting GlcNAc N-glycans Modulation. An Interplay with E-Cadherin

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Supplementary Figures of Chapter II

Figure S1: Effects of exogenous E-cadherin expression on the phosphoproteome profile.
Figure S2: Effects of stimulation of Mock-transfected cells with insulin and IGF-I on the phosphorylation of tyrosine kinase receptors and downstream proteins**.**
Figure S3: Effects of stimulation of Mock-transfected cells with insulin and IGF-I on cell invasion.
Figure S4: Subcellular localization of E-cadherin and β-catenin of Mock-transfected cells stimulated with insulin and IGF-I.

Figure S1

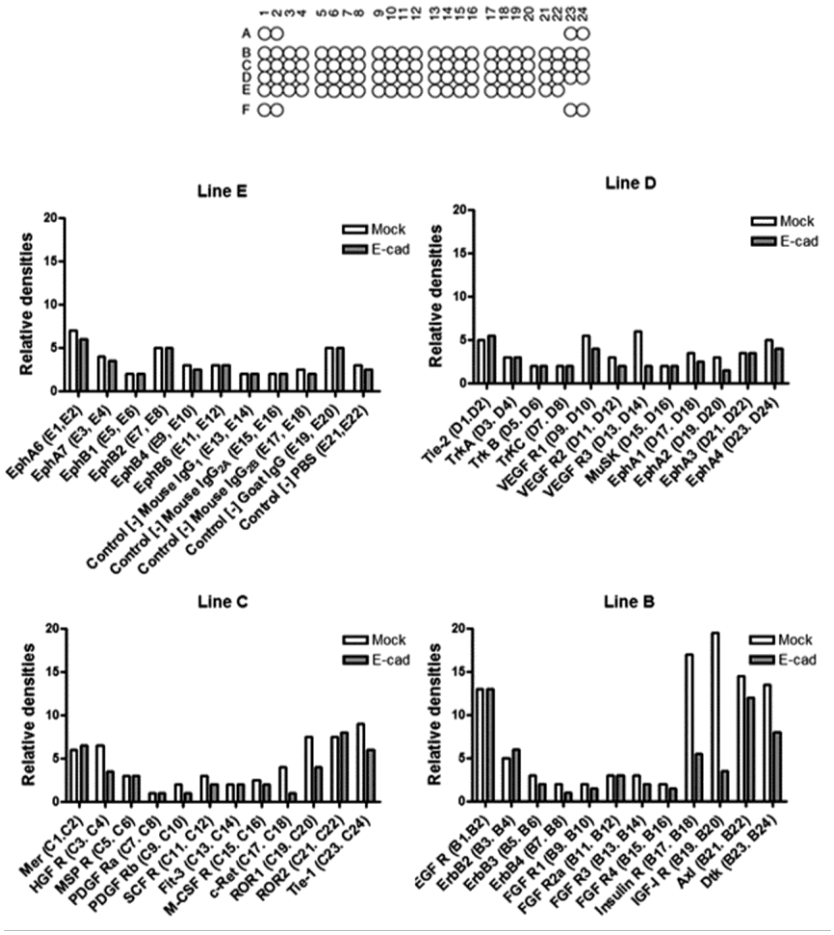

Figure S1. Effects of exogenous E-cadherin expression on the phosphoproteome profile. Total cell lysates from MDA-MB-435+mock and MDA-MB-435+E-cad were obtained and analyzed by Phospho-RTK array using 300 µg of proteins. The phosphor-RTK coordinates are shown on the top of figure illustrating the localization of the spots containing immobilized antibodies on the nitrocellulose membrane. The bar graphs show the relative densities of black dots. The most pronounced changes are observed in IR (coordinates B17 and B18) and IGF-IR (coordinates B19 and B20).

Figure S2

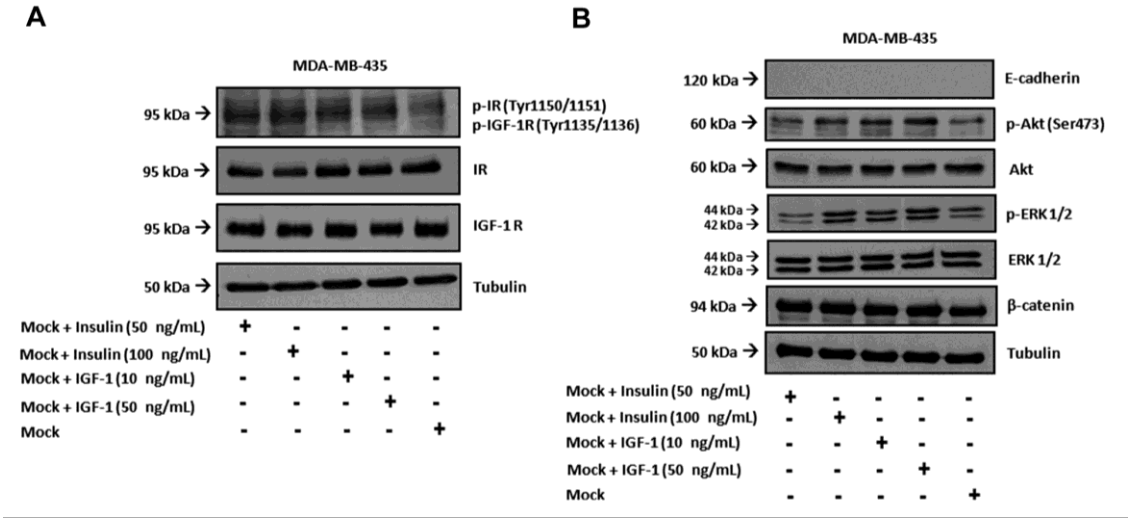

Figure S2. Effects of stimulation of Mock-transfected cells with insulin and IGF-I on the phosphorylation of tyrosine kinase receptors and downstream proteins. (A, B) Total cell lysates from MDA-MB-435+mock cells and MDA-MB-435+mock stimulated (24h) with insulin or IGF-1 were obtained and analyzed by Western-blot for phospho-IR(Tyr1150-51)/phospho-IGF-IR(Tyr1135-36), IR, IGFR, Akt, phospho-Akt (Ser 473), ERK 1/2, phospho-ERK 1/2, β-catenin and E-cadherin. Increased phosphorylation levels of IR, IGF-IR, ERK 1/2 and Akt were observed after stimulation with insulin or IGF-I. Tubulin was used as a loading control.

Figure S3

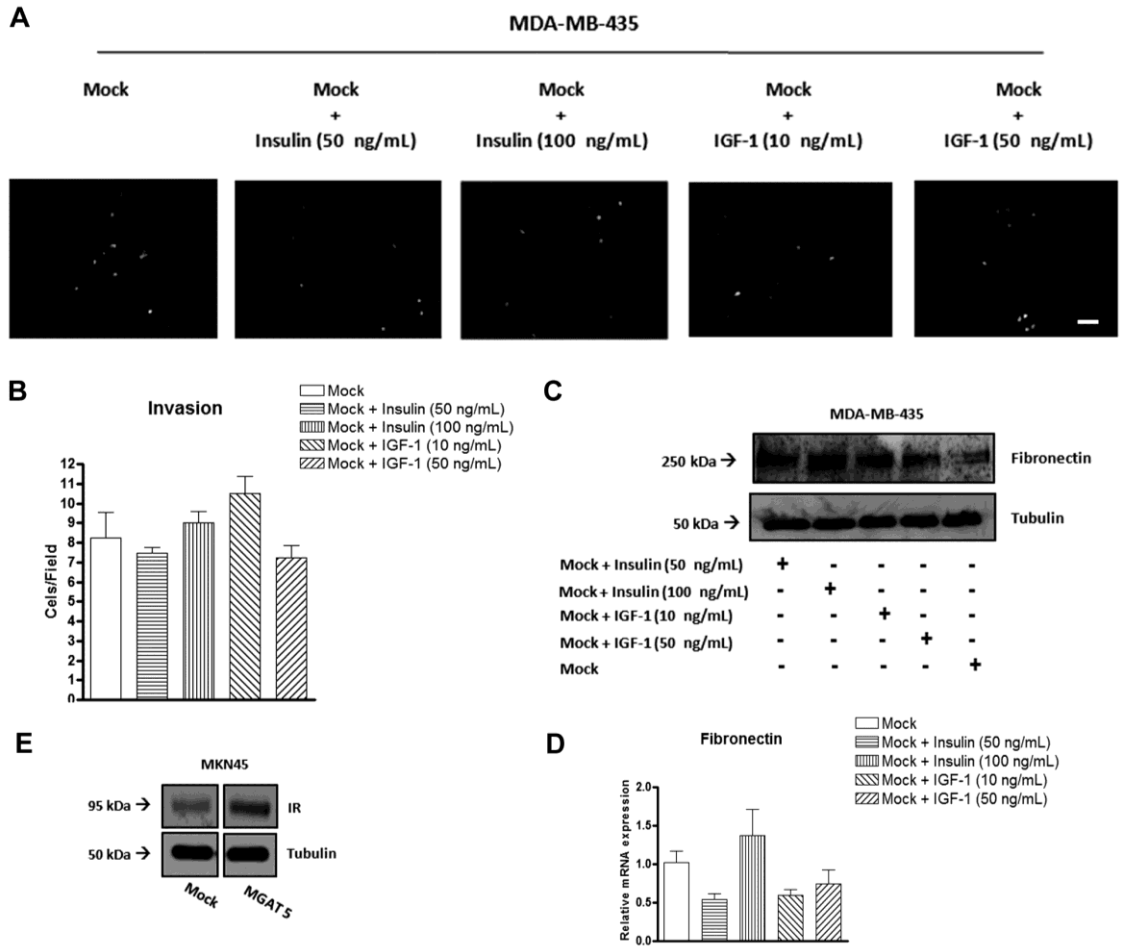

Figure S3. Effects of stimulation of Mock-transfected cells with insulin and IGF-I on cell invasion. (A) Representative images of cell invasion through Matrigel using 8 mm pore of a polycarbonate membrane. Nuclei were stained with DAPI. No significant differences were observed on cellular invasion upon insulin and IGF-I stimulation of mock-transfected cells. (B) The bar graph shows the amount of cells/field. Effects of stimulation with insulin and IGF-I on the fibronectin protein and mRNA expression levels, respectively. (C) and (D) A slight increase of fibronectin protein expression levels were observed after stimulation with insulin or IGF-I, however, no significant changes were observed at the mRNA transcription levels after stimulation of MDA-MB-435+E-mock cells with insulin and IGF-I .Effects of overexpression of *MGAT5* on the IR expression levels of MKN45 cell line. (E) Total cell lysates from MKN45+mock and MKN45+*MGAT5* were obtained and analyzed by Western blot for IR. An increased expression of IR were observed after overexpression of *MGAT5*. Tubulin was used as a loading control.

Figure S4

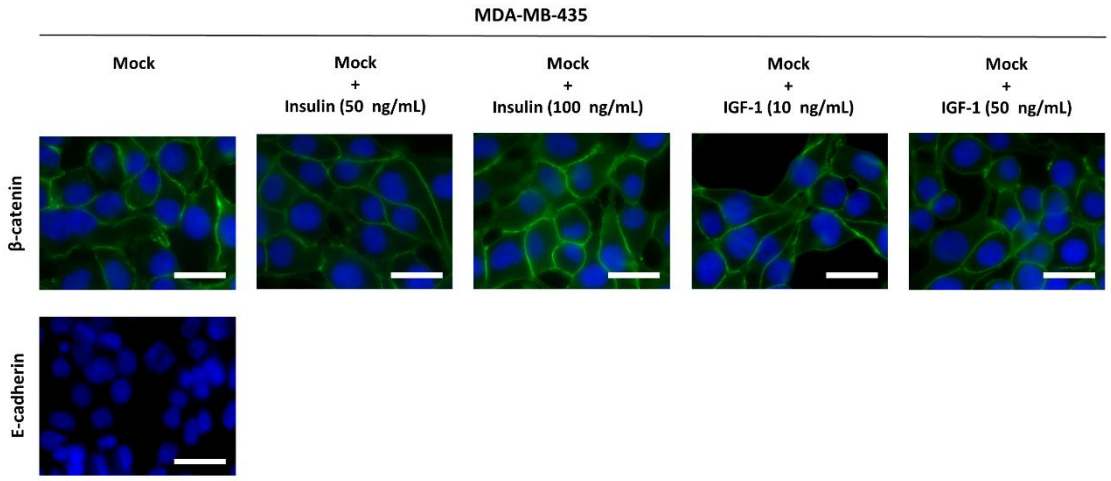

Figure S4. Subcellular localization of E-cadherin and β-catenin of Mock-transfected cells stimulated with insulin and IGF-I. Cell monolayers from MDA-MB-435+mock stimulated (24h) with insulin or IGF-1 were fixed and stained for E-cadherin, β-catenin and nucleus (DAPI). No significant differences were observed on the β-catenin subcellular localization after insulin or IGF-I stimulation. The representative images were obtained by fluorescence microscopy. Bar = 10 μm.

Appendix II

Preventing E-cadherin aberrant N-glycosylation at Asn-554 improves its critical function in gastric cancer.

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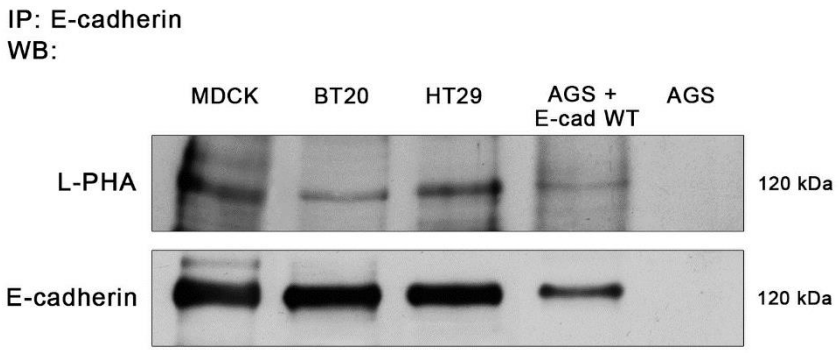
Supplementary Figures of Chapter III

Supplementary Figure 1: Evaluation of the branched N-glycosylation profile of E-cadherin mediated by GnT-V in different cell lines from different cellular origins.
Supplementary Figure 2: Relative abundance of the released N-glycans carried by E-cadherin and its respective mutants M123 and M234.
Supplementary Figure 3: Evaluation of the N-glycosylation profile of E- cadherin M1, M2, M3, and M4 in AGS cells.
Supplementary Figure 4: Evaluation of the impact of site-specific occupancy of Asn-554 (site 1) with complex type N-glycans on E-cadherin biological functions.
Supplementary Figure 5: Evaluation of the calcium binding effect on E-cadherin N-glycan mutant expression.
Supplementary Figure 6: Analysis of N-glycosylation profile mediated by GnT-V after *MGAT5* knockdown.

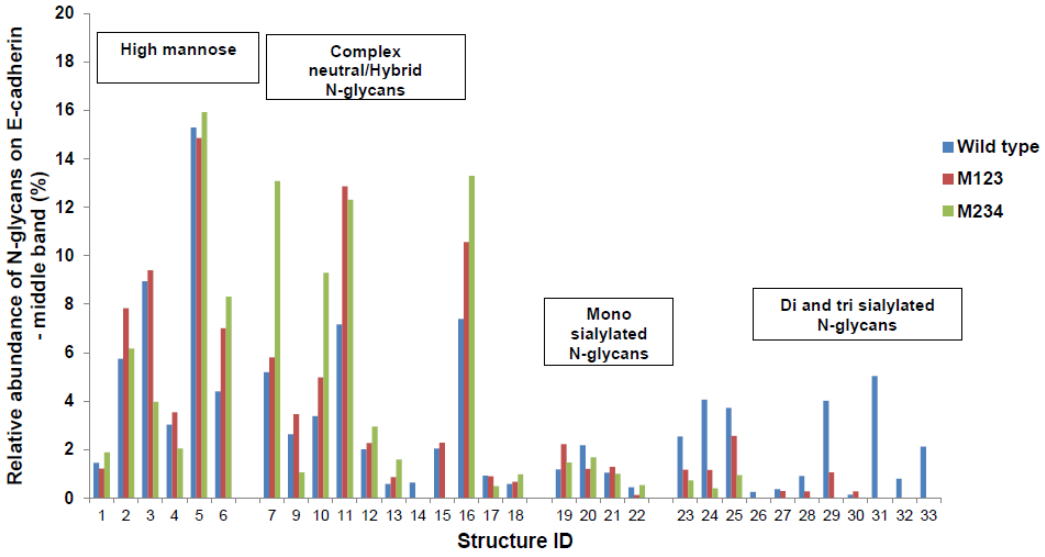
Supplementary Table 1: List of released N-glycans detected on E-cadherin WT and the respective mutants namely M123 and M234.
Supplementary Table 2: List of released N-glycans detected on E-cadherin WT and the respective mutants M123 and M234.

Supplementary Figure 1



Supplementary Figure 1. Evaluation of the branched N-glycosylation profile of E-cadherin mediated by GnT-V in different cell lines from different cellular origins. The pattern of branched glycosylation of E-cadherin comparing normal epithelial cells (MDCK), breast (BT20), colon (HT29) and gastric cancer (AGS) cells varies in a cell and tissue specific manner.

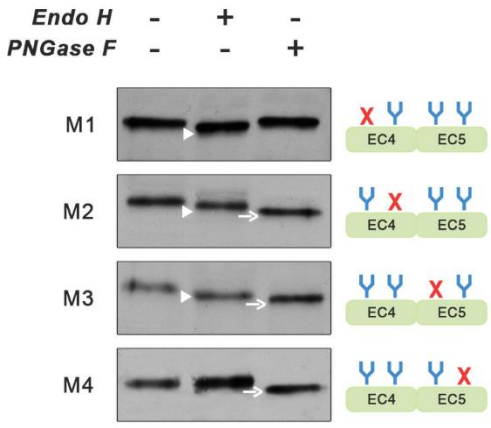
Supplementary Figure 2



Supplementary Figure 2. Relative abundance of the released N-glycans carried by E-cadherin and its respective mutants M123 and M234. Overview on the N-glycan distribution of E-cadherin WT (blue bars), M123 (brown bars) and M234 (green bars). The structure ID numbers associated with the respective structures is found in Supplementary Table 1 and the respective relative intensities in Supplementary Table 2. The data clearly shows that despite the fact that the overall amount of the high mannose type structures

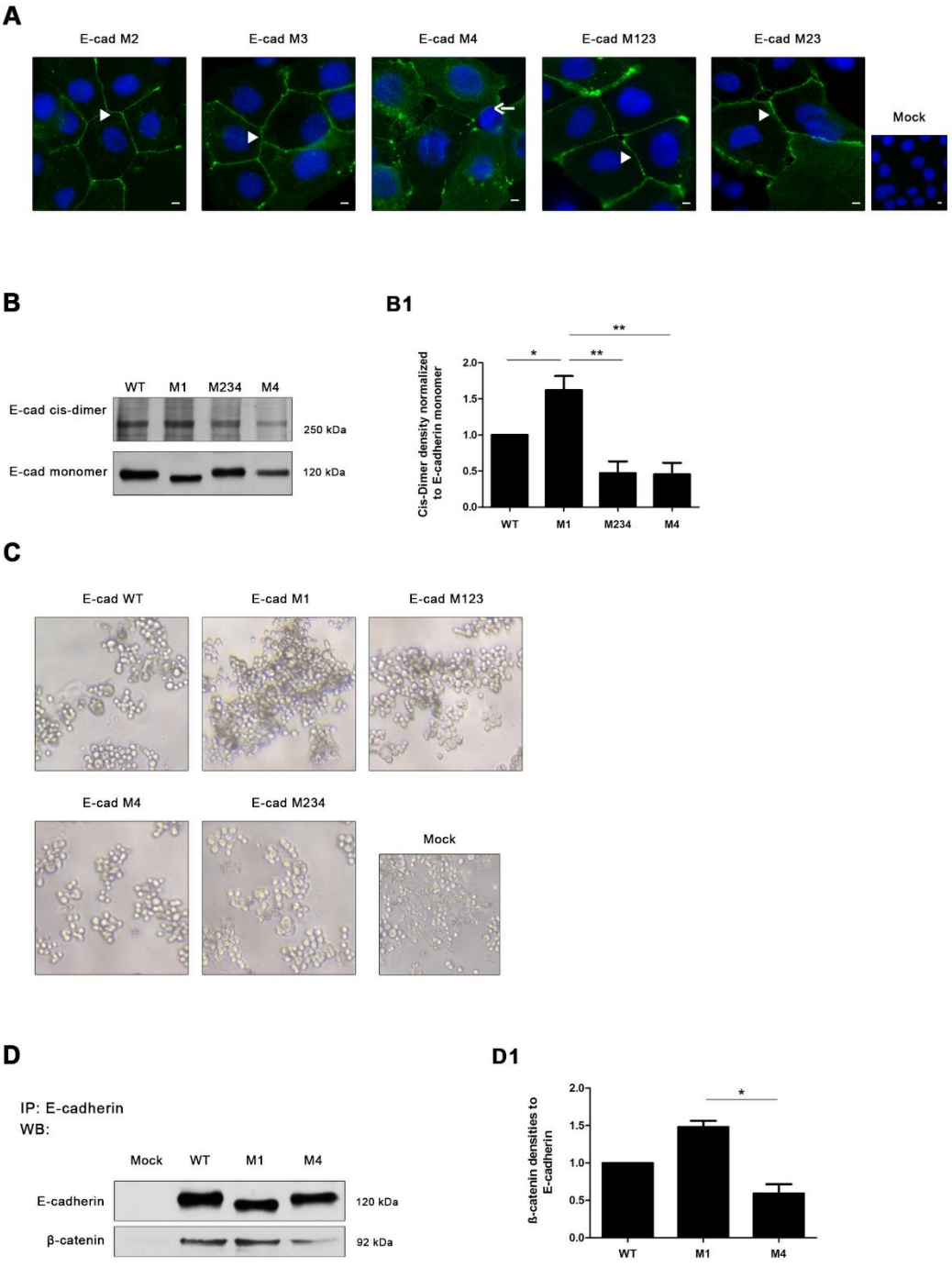
remains unaltered, E-cadherin WT and M123 contain increased amounts of complex type di- and triantennary structures, which were not detectable in M234. Furthermore, M234 also showed an increased level of Man9 type structure, but reduced levels of processed high mannose type structures.

Supplementary Figure 3



Supplementary Figure 3. Evaluation of the N-glycosylation profile of E- cadherin M1, M2, M3, and M4 in AGS cells. E-cadherin M2 and M3 were sensitive to *Endo H* (arrowhead) and *PNGase F* (arrow) being modified with high mannose, hybrid, and complex-type N-glycans. E-cadherin M4 was predominantly *PNGase F* sensitive (arrow) while E-cadherin M1 was predominantly *Endo H*-sensitive (arrowhead).

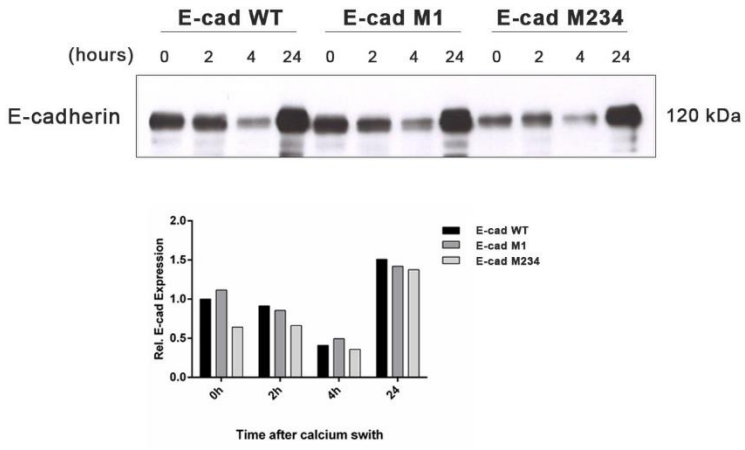
Supplementary Figure 4



Supplementary Figure 4. Evaluation of the impact of site-specific occupancy of Asn-554 (site 1) with complex type N-glycans on E-cadherin biological functions (A) Immunofluorescence analysis of E-cadherin M2, M3, M123, and M23 N-glycan mutants displaying a membrane pattern of E-cadherin expression (arrowhead), and E-cadherin M4 exhibiting an aberrant pattern of E-cadherin expression as evidenced by a clear cytoplasmic

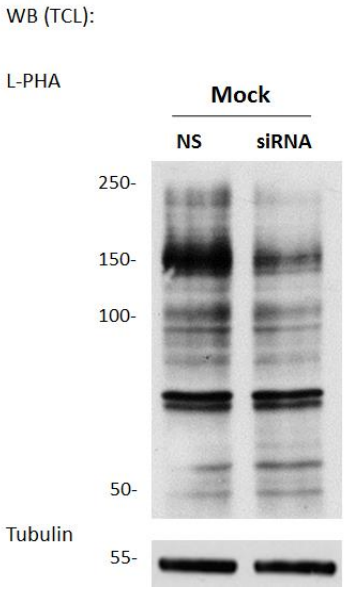
staining (arrow). **(B)** Evaluation of cis-dimer formation of E-cadherin WT, M1, M234, and M4. **(B1)** *Bar graphs*. Amounts of E-cadherin cis-dimer were determined from the ratio of densities of E-cadherin cis-dimer/ E-cadherin monomer (without BS3), and normalization to E-cadherin WT. Results are described as mean ±standard deviation of two independent experiments. **(C)** Evaluation of the cell-cell aggregation capacity of E-cadherin WT, M1, M123, M4, and M234. **(D)** E-cadherin M4 mutant (with site 1 available) displays a decreased interaction between E-cadherin and β-catenin, as was observed for M234 mutant. **(D1)** *Bar graphs*. Amounts of association were determined from the ratios of densities of β- catenin after normalization to E-cadherin.

Supplementary Figure 5



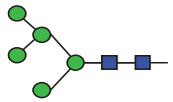
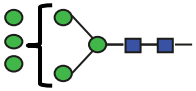
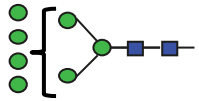
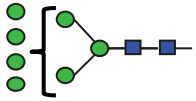
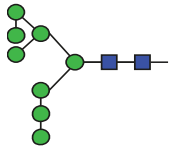
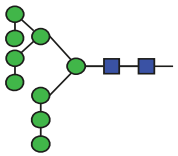
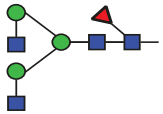
Supplementary Figure 5. Evaluation of the calcium binding effect on E-cadherin expression among the different N-glycosylation forms. Mutagenesis of different N-glycosylation sites of E-cadherin did not interfere with the calcium binding property. After 24h of calcium chelation (with EGTA), the expression of E-cadherin was completely recovered in all E-cadherin N-glycosylation forms, supporting that the calcium binding property is independent of E-cadherin glycosylation.

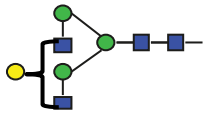
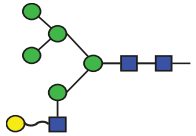
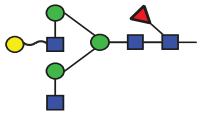
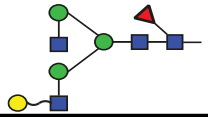
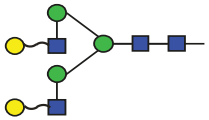
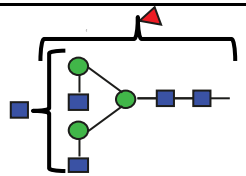
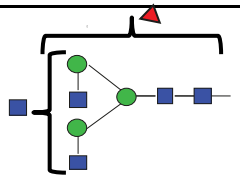
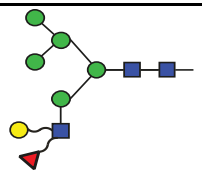
Supplementary Figure 6

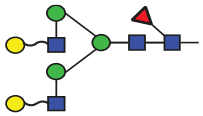
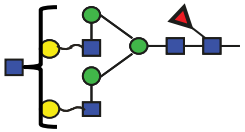
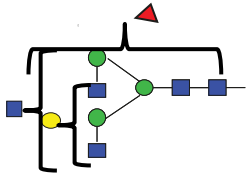
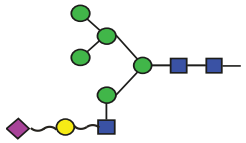
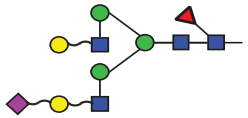
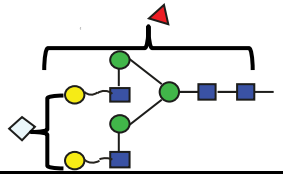
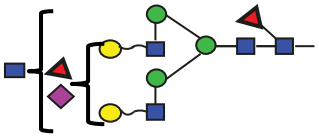
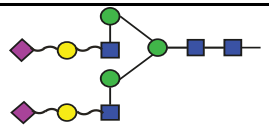


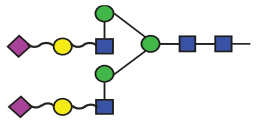
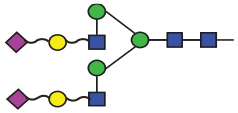
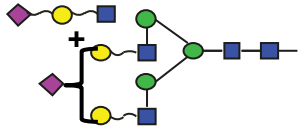
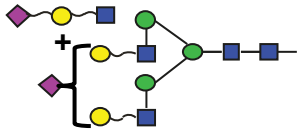
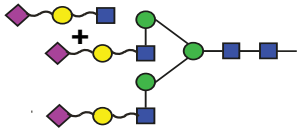
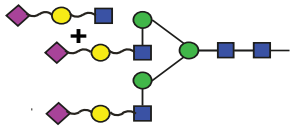
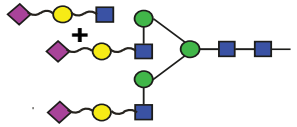
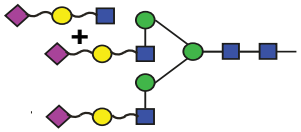
Supplementary Figure 6. Analysis of branched N-glycosylation profile of AGS cells after *MGAT5* knockdown. Lectin blot analysis of GnT-V product on total cell lysate from AGS Mock cells before (NS) and after *MGAT5* knockdown (siRNA). A decreased L-PHA reactivity after *MGAT5* knockdown was observed.

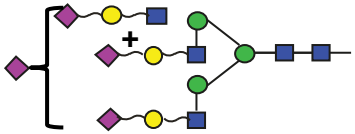
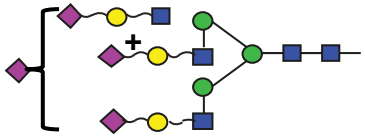
Supplementary Table 1- List of released N-glycans detected on E-cadherin WT and the respective mutants namely M123 and M234.

ID	theor. [M-H]-	Core	Hex	HexNAc	Fuc	NeuAc	NeuGc	Structure	Category	Comments
1	1235,44	1	2	0	0	0	0		Oligomannose	MS/MS
2	1397,49	1	3	0	0	0	0		Oligomannose	MS/MS
3	1559,55	1	4	0	0	0	0		Oligomannose	MS/MS
4	1559,55	1	4	0	0	0	0		Oligomannose	MS/MS
5	1721,60	1	5	0	0	0	0		Oligomannose	MS/MS
6	1883,65	1	6	0	0	0	0		Oligomannose	MS/MS
7	1463,55	1	0	2	1	0	0		Complex neutral	MS/MS

8	1479,55	1	1	2	0	0	0		Complex neutral	no MS/MS
9	1600,59	1	3	1	0	0	0		Hybrid	MS/MS
10	1625,61	1	1	2	1	0	0		Complex neutral	MS/MS
11	1625,61	1	1	2	1	0	0		Complex neutral	MS/MS
12	1641,60	1	2	2	0	0	0		Complex neutral	MS/MS
13	1666,63	1	0	3	1	0	0			no MS/MS
14	1666,63	1	0	3	1	0	0			no MS/MS
15	1746,63	1	3	1	1	0	0		Hybrid	MS/MS

16	1787,66	1	2	2	1	0	0		Complex neutral	MS/MS
17	1990,74	1	2	3	1	0	0			MS/MS
18	1828,69	1	1	3	1	0	0			no MS/MS
19	1891,67	1	3	1	0	1	0		Hybrid	MS/MS
20	1932,70	1	2	2	0	1	0		Complex sialylated	MS/MS
21	2094,75	1	2	2	1	0	1		Complex sialylated	no MS/MS
22	2427,89	1	2	3	2	1	0		Complex sialylated	no MS/MS
23	2223,79	1	2	2	0	2	0		Complex sialylated	MS/MS

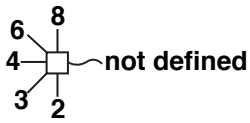
24	2223,79	1	2	2	0	2	0		Complex sialylated	MS/MS
25	2223,79	1	2	2	0	2	0		Complex sialylated	MS/MS
26	2588,92	1	3	3	0	2	0		Complex sialylated	no MS/MS
27	2588,92	1	3	3	0	2	0		Complex sialylated	no MS/MS
28	2880,02	1	3	3	0	3	0		Complex sialylated	no MS/MS
29	2880,02	1	3	3	0	3	0		Complex sialylated	MS/MS
30	2880,02	1	3	3	0	3	0		Complex sialylated	no MS/MS
31	2880,02	1	3	3	0	3	0		Complex sialylated	MS/MS

32	3171,11	1	3	3	0	4	0		Complex sialylated	no MS/MS
33	3171,11	1	3	3	0	4	0		Complex sialylated	no MS/MS

Legend

Mannose Galactose N-Acetylglucosamine N-Glycolylneuraminic acid

Fucose N-Acetylneuraminic acid



Supplementary Table 2- List of released N-glycans detected on E-cadherin WT and the respective mutants M123 and M234.

Structure ID	Wild type	M123	M234	Wild type	M123	M234			1,01	911,34	162,1	203,08	146,06	291,1			
	rt	rt	rt	m/z	m/z	m/z	average m/z	z	[M-H]-	core	hex	HexNAc	Fuc	NeuAc	NeuGc	m/z theor	ΔM
	High Mannose																
1	32,0	31,9	32,1	1235,50	1235,49	1235,56	1235,52	1	1235,52	1	2	0	0	0	0	1235,44	-0,075
2	24,2	24,4	24,2	1397,59	1397,65	1397,48	1397,57	1	1397,57	1	3	0	0	0	0	1397,49	-0,079
2	24,2	24,2	24,2	698,26	698,28	698,28	698,27	2	1397,55	1	3	0	0	0	0	1397,49	-0,060
3	23,0	23,3	23,2	1559,66	1559,53	1559,51	1559,57	1	1559,57	1	4	0	0	0	0	1559,55	-0,019
4	24,2	24,4	24,4	1559,49	1559,45	1559,51	1559,48	1	1559,48	1	4	0	0	0	0	1559,55	0,064
3	23,0	23,1	23,3	779,29	779,30	779,32	779,30	2	1559,61	1	4	0	0	0	0	1559,55	-0,067
4	24,2	24,2	24,2	779,29	779,31	779,27	779,29	2	1559,59	1	4	0	0	0	0	1559,55	-0,040
5	23,2	23,3	23,2	860,32	860,31	860,34	860,32	2	1721,65	1	5	0	0	0	0	1721,60	-0,054
6	23,6	23,7	23,7	941,37	941,35	941,32	941,35	2	1883,70	1	6	0	0	0	0	1883,65	-0,048
	Complex neutral/Hybrid																
7	31,0	31,1	31,1	731,30	731,30	731,31	731,30	2	1463,61	1	0	2	1	0	0	1463,55	-0,062
8	27,2			739,30			739,30	2	1479,61	1	1	2	0	0	0	1479,55	-0,060
9	27,5	27,5	27,6	799,80	799,80	799,75	799,78	2	1600,57	1	3	1	0	0	0	1600,57	0,000
10	33,3	33,4	33,4	812,34	812,32	812,34	812,33	2	1625,67	1	1	2	1	0	0	1625,61	-0,069
11	34,0	34,0	34,0	812,31	812,31	812,33	812,32	2	1625,64	1	1	2	1	0	0	1625,61	-0,035
12	29,7	29,6	29,7	820,30	820,34	820,32	820,32	2	1641,65	1	2	2	0	0	0	1641,60	-0,047
13	21,9	21,8	21,9	832,81	832,82	832,83	832,82	2	1666,65	1	0	3	1	0	0	1666,63	-0,016
14	29,0			832,84			832,84	2	1666,69	1	0	3	1	0	0	1666,63	-0,056
15	24,9	24,9		872,81	872,82		872,82	2	1746,64	1	3	1	1	0	0	1746,63	-0,006
16	36,2	36,0	36,2	893,36	893,32	893,37	893,35	2	1787,71	1	2	2	1	0	0	1787,66	-0,049
17	25,7	25,7	25,7	994,85	994,83	994,79	994,82	2	1990,65	1	2	3	1	0	0	1990,74	0,083
18	24,4	24,4	24,4	913,83	913,87	913,82	913,84	2	1828,69	1	1	3	1	0	0	1828,69	-0,003
	Mono sialylated																
19	28,9	29,0	29,3	945,39	945,32	945,40	945,37	2	1891,75	1	3	1	0	1	0	1891,67	-0,078
20	30,8	31,4	31,2	965,87	965,84	965,77	965,83	2	1932,66	1	2	2	0	1	0	1932,70	0,035
21	36,3	36,6	36,7	1046,84	1046,83	1046,85	1046,84	2	2094,69	1	2	2	1	0	1	2094,75	0,061
22	25,9			1213,42			1213,42	2	2427,85	1	2	3	2	1	0	2427,89	0,043
	Di- and tri-sialylated																
23	27,0	27,5	27,3	1111,40	1111,39	1111,46	1111,42	2	2223,84	1	2	2	0	2	0	2223,79	-0,050
24	32,6	33,2	33,5	1111,42	1111,38	1111,39	1111,40	2	2223,80	1	2	2	0	2	0	2223,79	-0,010
25	41,7	42,6	42,8	1111,38	1111,42	1111,4	1111,40	2	2223,81	1	2	2	0	2	0	2223,79	-0,016
26	36,5			1293,95			1293,95	2	2588,91	1	3	3	0	2	0	2588,92	0,016
27	43,2			1293,90			1293,90	2	2588,81	1	3	3	0	2	0	2588,92	0,116

28	27,5	27,6		1439,55	1439,49		1439,52	2	2880,05	1	3	3	0	3	0	2880,02	-0,029
29	35,5	35,7		1439,51	1439,49		1439,50	2	2880,01	1	3	3	0	3	0	2880,02	0,011
30	44,5	44,4		1439,55	1439,53		1439,54	2	2880,09	1	3	3	0	3	0	2880,02	-0,069
31	52,2			1439,56			1439,56	2	2880,12	1	3	3	0	3	0	2880,02	-0,099
28	27,5			959,34			959,34	3	2880,04	1	3	3	0	3	0	2880,02	-0,017
29	35,7	35,7		959,28	959,37		959,33	3	2879,99	1	3	3	0	3	0	2880,02	0,028
31	52,0			959,36			959,36	3	2880,10	1	3	3	0	3	0	2880,02	-0,077
32	35,7			1056,32			1056,32	3	3170,98	1	3	3	0	4	0	3171,11	0,139
33	54,5			1056,35			1056,35	3	3171,07	1	3	3	0	4	0	3171,11	0,049

Appendix III

O-Mannosylation and N-glycosylation: two coordinated mechanisms regulating the critical functions of E-cadherin in cancer.

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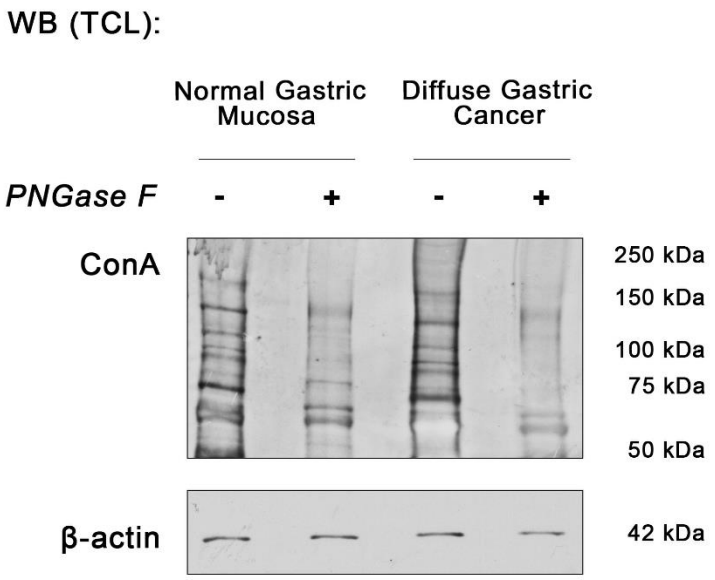
Supplementary Figures of Chapter IV

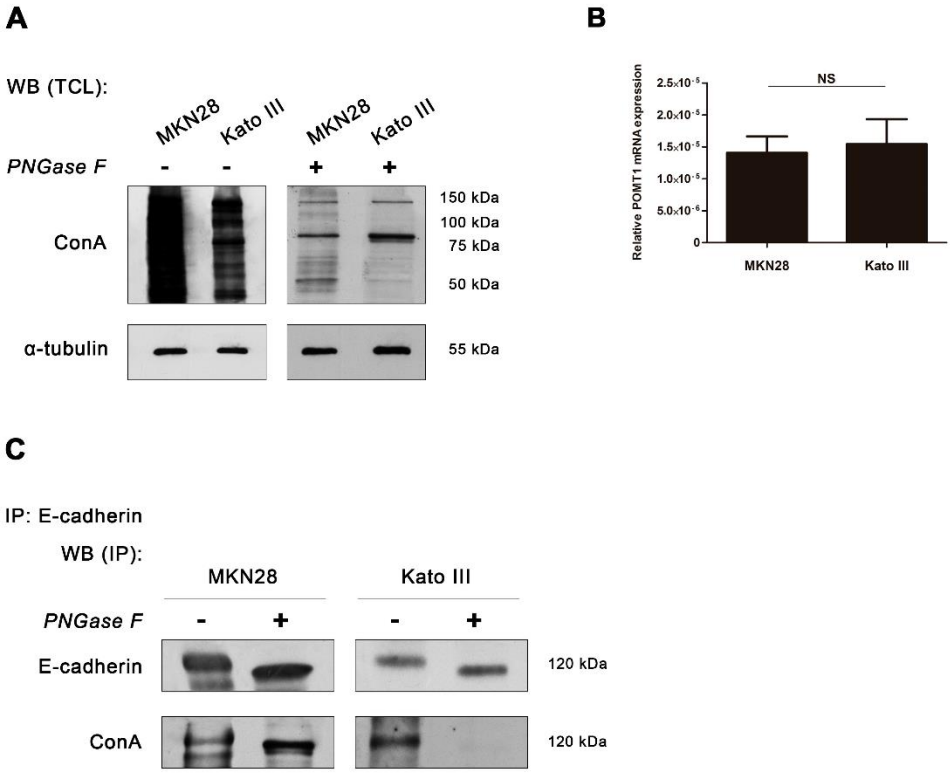
Supplementary Figure 1: Profile of O-mannosyl glycans on stomach tissue lysate.
Supplementary Figure 2: Evaluation of O-mannosyl glycans profile, and particularly on E-cadherin in MKN28 and Kato III cells.
Supplementary Figure 3: Evaluation of PNGase F efficiency through mobility shift of E-cadherin band.
Supplementary Figure 4: Evaluation of mRNA transcripts of *POMT2* and *MGAT5* in different backgrounds

Supplementary Figure 1



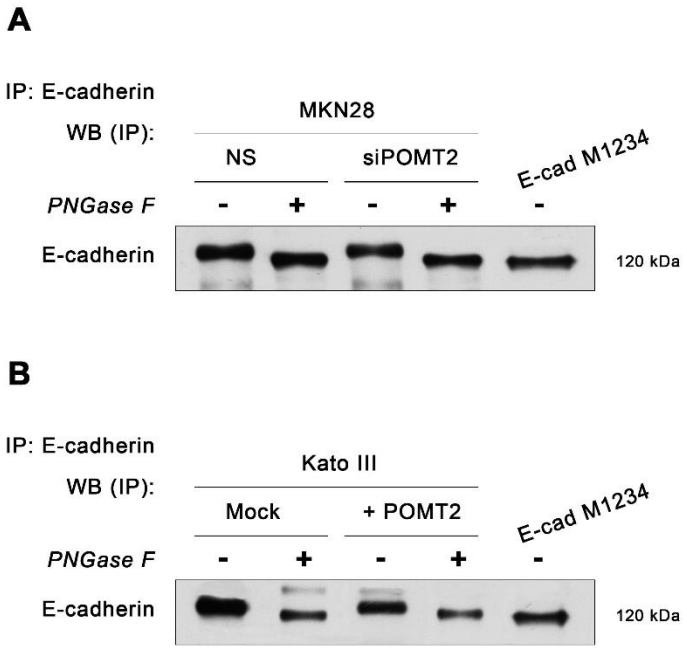
Supplementary Figure 1. Profile of O-mannosyl glycans on stomach tissue lysate. To evaluate the expression of O-mannosyl glycans, we performed to digestion of total tissue lysate with PNGase F to remove N-glycans, followed by lectin blotting using Con A lectin (which binds to α-mannose residues from O-Man and N-glycoproteins). As expected, PNGase F treatment resulted in a decrease in the reactivity to Con A lectin. Regarding the expression of O-mannosyl glycans (detected by treatment with PNGase F + Con A lectin blotting), diffuse gastric carcinoma displayed a decreased O-mannosylation profile.

Supplementary Figure 2



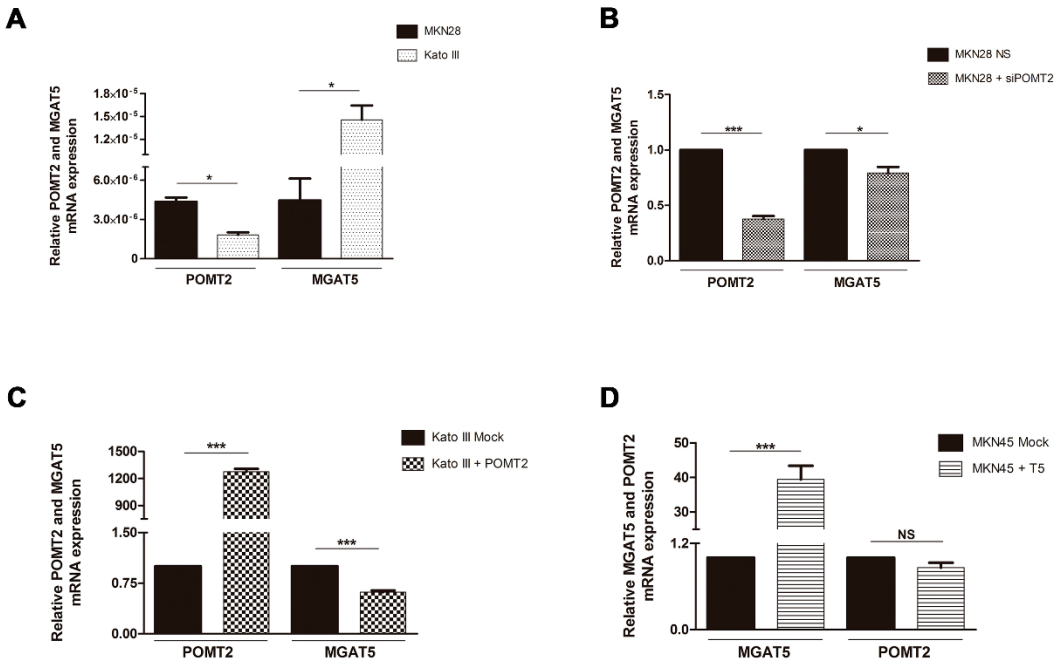
Supplementary Figure 2. Evaluation of O-mannosyl glycans profile, and particularly on E-cadherin in MKN28 and Kato III cells. (A) O-mannosyl glycans profile. α-linked mannose residues present in the core N-oligosaccharide were removed by PNGase F digestion and Con A- mannose-binding lectin blotting was performed to evaluate the expression of O-mannosyl glycans from O-mannosylated proteins. The reactivity to Con A lectin decreased after removal of N-glycans. Note that both represented lectin blots corresponded to the same exposure time. (B) POMT1 mRNA expression. No significant differences were verified in the transcript levels of POMT1 in MKN28 and Kato III cells. Results are described as mean ± s.d of three independent biological replicates. (C) O-mannosylation of E-cadherin in MKN28 and Kato III cell lines. E-cadherin immunoprecipitated from MKN28 cells exhibited a positive reactivity to Con A lectin (with and without PNGase F treatment). Regarding Kato III cells, E-cadherin immunoprecipitated showed a significant lower reactivity to Con A lectin after removal of N-glycans. Note that both represented lectin blots corresponded to the same exposure time.

Supplementary Figure 3



Supplementary Figure 3. Evaluation of PNGase F efficiency through mobility shift of E-cadherin band. E-cadherin immunoprecipitated after (A) POMT2 knockdown in MKN28 or (B) POMT2 overexpression in Kato III cells was treated with PNGase F enzyme to release the N-glycans, and the mobility shift resulting this treatment was compared with E-cadherin N-glycan naked (E-cadherin M1234).

Supplementary Figure 4



Supplementary Figure 4. Evaluation of mRNA transcripts of *POMT2* and *MGAT5* in four different backgrounds: (A1) MKN28 versus Kato III cells; after modulation of POMT2 expression either by (B1) knockdown or by (C1) overexpression, and (D1) after overexpression of *MGAT5* in MKN45 cell line. The relative POMT2 and *MGAT5* mRNA expression of MKN28 + siPOMT2, Kato III + POMT2 and MKN45 + T5 cells are expressed as the fold increase, compared with non-silencing cells or mock cells, respectively, which was taken as 1. The data presented is referred to three independent biological replicates.